REMARKS

In this Amendment, claims 1-4, 8, 20, and 28 are amended. After entry of this Amendment, claims 1-8, 20, 21 and 28 are all the claims pending in the application.

Claims 1 and 20 have been amended for clarity.

Dependent claim 2 has been amended to be consistent with claim 1, and is supported by original claim 1.

Claims 3 and 4 have also been amended for clarity, these amendments being supported by the specification at the paragraph bridging pages 28 and 29.

Claim 8 has been amended for clarity. The amendment to part (d) -- a cytokinin receptor "having at least one transmembrane region but fewer transmembrane regions than wild-type cytokinin receptor" -- is supported by the specification at page 22, lines 1-15. The amendment to part (h)-- a chimera-type cytokinin receptor comprising "extracellular regions, transmembrane regions and histidine kinase regions, all of which are derived from the same cytokinin receptor, and receiver regions which are not derived from said same cytokinin receptor"-- is supported by the specification at page 25, lines 19-23. The amendment to part (h) -- "and washing in the presence of 0.1 X SSC and 0.5% SDS at 68 °C for 30 minutes" -- is supported by the specification at page 16.

Claim 28 has likewise been amended to recite "and washing in the presence of 0.1 X SSC and 0.5% SDS at 68 °C for 30 minutes," and therefore is also supported by the specification at page 16.

No new matter has been introduced.

Entry of this Amendment is respectfully requested.

Response to Claim Rejections Under 35 USC §112, First Paragraph

At page 2 of the Office Action, the Examiner rejects claim 8 under 35 USC §112, first paragraph, because the specification allegedly does not enable one of skill in the art to measure the activity of the cytokinin receptors recited in part (i) of claim 8.

Specifically, the Examiner believes that deletion, substitution or addition of one or a plurality of amino acids may completely eliminate the histidine kinase activity or introduce unknown function(s) to the cytokinin receptor.

This rejection is respectfully traversed.

The cytokinin receptor recited in part (i) of claim 8 is a ***"cytokinin receptor"*** which requires that it have cytokinin receptor activity. Part (i) of claim 8 does not encompass a protein having one or more amino acid deletions, substitutions or additions that eliminate the histidine kinase activity, as the Examiner contends.

In addition, the cytokinin receptor of part (i) of claim 8 is encoded by a polynucleotide "that hybridizes under stringent conditions to a polynucleotide represented by the nucleotide sequence selected from the group consisting of SEQ ID Nos: 1, 3, and 5, and wherein said stringent conditions comprise hybridization at 6 X SSC at 65 °C and washing in the presence of 0.1 X SSC and 0.5% SDS at 68 °C for 30 minutes." Methods for obtaining such a polynucleotide are disclosed in the specification from page 15, line 17 to page 17, line 15.

Accordingly, part (i) of claim 8 is enabled by the specification, and withdrawal of this rejection is respectfully requested.

Response to Claim Rejections Under 35 USC §112, Second Paragraph

At page 4 of the Office Action, the Examiner rejects claims 1-8, 20, 21 and 28 under 35 USC §112, second paragraph, as being indefinite.

(1) The Examiner contends that the language measuring an "existence" to thereby determine a "level" of signal transduction renders claims 1 and 20 indefinite, because measuring an "existence" will not determine a "level" of signal transduction.

Claims 1 and 20 have been amended to recite: "(2) determining an existence or level of intracellular signal transduction from said cytokinin receptor."

(2) The Examiner contends that claim 20 is incomplete because the preamble says "detecting" agonist activity, but the claim allegedly does not achieve the goal of "detecting" activity.

Claim 20 has been amended to recite: "A method for determining agonist-activity." Claim 1, which is not rejected as being indefinite, uses this same language.

(3) The Examiner contends that claims 8 and 28 are indefinite, because the scope of hybridizing polynucleotides will depend on time and temperature of hybridization, as well as on the washing conditions.

Claims 8 and 20 have been amended to also recite the washing conditions.

(4) The Examiner contends that claim 8, part (d), is indefinite because the term "a partially transmembrane region-deleted type cytokinin receptor" is allegedly unclear.

The Examiner further believes that claim 8, part (h), is indefinite because it is not clear to the Examiner what constitutes "a chimera-type cytokinin receptor," other than "heterogeneous receiver regions."

The Examiner further believes that the term "heterogeneous receiver region" is unclear.

The Examiner contends that claim 8, part (i), is indefinite because it allegedly depends from parts (d) and (h).

Initially, the Examiner is reminded that the present claims are accompanied by an entire specification providing descriptive support for the claims. To the extent that the Examiner does not readily understand certain terms used in the claims, and desires a further description, the Examiner is invited to review the specification which describes these terms in detail.

Nevertheless, claim 8, part (d), has been amended to recite "a cytokinin receptor having at least one transmembrane region but fewer transmembrane regions than wild-type cytokinin receptor." See page 22 of the specification. To the extent that the Examiner still believes that part (d) is indefinite, the Examiner is invited to suggest preferred language. See MPEP §2173.02.

Claim 8, part (h), has been amended to recite: "extracellular regions, transmembrane regions and histidine kinase regions, all of which are derived from the same cytokinin receptor, and receiver regions which are not derived from said same cytokinin receptor." See page 25 of the specification. To the extent that the Examiner still believes that part (d) is indefinite, the Examiner is invited to suggest preferred language. See MPEP §2173.02.

Claim 8, part (i), has been amended to include washing conditions. The Examiner is also requested to note that claim 8, part (i), does not depend from parts (d) and (h).

(5) The Examiner contends that the term "lowered" in claims 3 and 4 is a relative term, which allegedly renders these claim indefinite. The Examiner believes that the term "lowered" is not defined by the claim, and that the specification does not provide a standard for ascertaining the "requisite degree."

Initially, the Examiner is requested to note that the verb "lower***ed***" is not an ambiguous term.

Nevertheless, claim 3 has been amended to recite: "wherein said host cell is improved so as to have a lower histidine kinase activity lower than before the improvement." Claim 4 already recites that the histidine kinase activity was lowered "by a defect in one or more histidine kinase genes."

The Examiner is again reminded that the specification provides descriptive support for the claim. The Examiner's attention is specifically directed to pages 28 and 29 of the specification. To the extent that the Examiner still believes that claim 3 or 4 is indefinite, the Examiner is invited to suggest preferred language. See MPEP §2173.02.

Withdrawal of the indefiniteness rejections is respectfully requested.

Response to Claim Rejections Under 35 USC §102

(1) At page 5 of the Office Action, the Examiner rejects claims 1, 2, 20 and 21 under 35 USC §102(b) as being anticipated by Kakimoto, *Science* 274:982-985 (1996).

Specifically, the Examiner contends that Kakimoto discloses the cloning of a cytokinin receptor gene into wild type calli and teaches screening assays for cytokinin activity at Figs. 1 and 3. The Examiner states that Kakimoto teaches that the CKI1 gene is a histidine kinase homolog implicated in cytokinin signal transduction, and that overexpression of CKI1 in plants results in characteristic effects of cytokinin action, although its role in 'cyokinin signal transduction is not clear. According to the Examiner, Kakimoto teaches screening for cytokinin-independent mutants, and teaches transformation of wild-type calli with CKI1 cDNA.

This rejection is respectfully traversed.

Kakimoto, *Science* (1996) does not teach that the CKI1 has cytokinin activity, as would be necessary to anticipate the present claims. As acknowledged by the Examiner, Kakimoto teaches that "the role of CKI1 in cytokinin signal transduction is not clear" (Kakimoto, page 984).

Further, Kakimoto in *Current Opinion in Plant Biology*, 1:399-403 (1998) (which was disclosed in the IDS filed December 13, 2002), and which was published two years after the cited reference, teaches:

> [i]t is attractive to ***hypothesize*** that CKI1 is a cytokinin receptor. Because the CKI gene was identified by activation tagging, it is important to know the effects of reduction or elimination of the CKI1 gene expression.
>
> ***In addition, to test whether the CKI1 protein binds cytokinin will be critically important.*** (see Kakimoto, *Current Opinion in Plant Biology* at page 400).

Further, Pischke et al., *Proc. Natl. Acad. Sci. USA* 99:15800-15805 (2002), a copy of which is attached to this response, teaches:

> ***Cytokinin is unlikely to be the ligand for CKI1 for several reasons***. The lack of homology between the putative extracellular domain of CKI1 and that of the cytokinin receptor CRE1, and its two close homologues AHK2 and AHK3, argues against a role for CKI1 as a cytokinin receptor. Membranes isolated from fission yeast expressing CKI1 do not bind radiolabeled cytokinin. Furthermore, the expression of CKI1 in *Escherichia coli* lacking the histidine kinase RcsC and in *Arabidopsis* protoplasts coexpressing a cytokinin-inducible reporter gene has been shown, in both cases, to activate histidine-kinase-signaling pathways in a constitutive, rather than cytokinin-dependent, manner. (see page 1505 of Pischke et al; citations omitted)

Further still, Higuchi et al., *Proc. Natl. Acad. Sci. USA* 101:8821-8826 (2004) teaches at page 8821:

> CKI1 was first identified as a gene that induces ***constitutive*** cytokinin responses when overexpressed in callus tissue. However, ***all subsequent efforts to detect cytokinin receptor activity of CKI1 have yielded negative results***.

Copies of Kakimoto, *Current Opinion in Plant Biology*, 1:399-403 (1998), Pischke et al., *Proc. Natl. Acad. Sci. USA* 99:15800-15805 (2002), and Higuchi et al., *Proc. Natl. Acad. Sci. USA* 101:8821-8826 (2004), are attached to this Amendment.

Thus, Kakimoto, *Science* 274: 982-985 (1996) does not disclose a cytokinin receptor activity of the CKI1 gene, and thus does not anticipate claims 1, 2, 20 and 21. Accordingly, withdrawal of this rejection is requested.

(2) At page 6 of the Office Action, the Examiner rejects claims 1-8, 20, 21 and 28 under 35 USC §102(a) as being anticipated by Inoue et al, *Nature* 409:1060-1063 (2001).

This rejection is respectfully traversed, because, in view of the attached Declarations Under 37 CFR §1.132, Inoue is not prior art to this application.

Specifically, Inoue et al, *Nature* (2001) describes the work of the present inventors. The co-inventors of the claimed invention, Tatsuo KAKIMOTO, Tsutomu INOUE and Masayuki HIGUCHI, are each listed as co-authors of Inoue et al., *Nature* (2001). The remaining authors, Yukari HASHIMOTO, Motoaki SEKI, Masatomo KOBAYASHI, Tomohiko KATO, Satoshi TABATA and Kazuo SHINOZAKI are listed as co-authors of Inoue et al. as a general matter of courtesy but are not co-inventors of the claimed subject matter.

Attached to this Amendment is a Declaration under 37 CFR §1.132 executed by Tatsuo Kakimoto, Tsutomu Inoue and Masayuki Higuchi, as well as three additional Declarations under 37 CFR §1.132 executed by: (1) Yukari Hashimoto; (2) Motoaki Seki, Masatomo Kobayashi, and Kazuo Shinozaki; and (3) Tomohiko Kato and Satoshi Tabata.

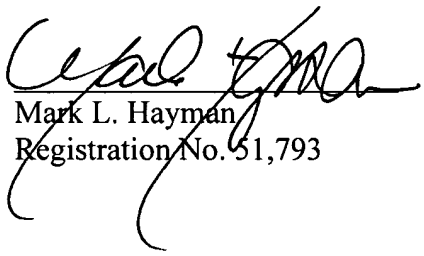
Accordingly, claims 1-8, 20, 21 and 28 are not anticipated by Inoue et al., and withdrawal of this rejection is respectfully requested.

Conclusion

In view of the above, reconsideration and allowance of this application are now believed to be in order, and such actions are hereby solicited. If any points remain in issue which the Examiner feels may be best resolved through a personal or telephone interview, the Examiner is kindly requested to contact the undersigned at the telephone number listed below.

The USPTO is directed and authorized to charge all required fees, except for the Issue Fee and the Publication Fee, to Deposit Account No. 19-4880. Please also credit any overpayments to said Deposit Account.

Respectfully submitted,



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Cytokinin signaling

Tatsuo Kakimoto

XP-001079011

Although cytokinin plays a central role in plant development, our knowledge of the biosynthesis, distribution, perception and signal transduction of cytokinin is limited. Recent molecular-genetic studies have, however, implicated involvement of a two-component system in cytokinin signal transduction. Furthermore, new mutants with altered cytokinin responses and genes involved in cytokinin signaling have been identified.

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Current Opinion in Plant Biology 1998, 1:399–403

http://biomednet.com/elecref/1369526600100399

Abbreviations

ACC	1-aminocyclopropane-1-carboxylate
ACS	ACC synthase
HPt	histidine-containing phosphotransfer

Introduction

Cytokinin was discovered as a factor that induces cell division in the presence of auxin [1]. Soon after this discovery, Skoog and Miller demonstrated that auxin and cytokinin determine the fate of the cells in tissue culture [2]. The drastic effects of cytokinin suggest that cytokinin plays a central role in controlling cell division and cell-fate in plants. Furthermore, cytokinin has diverse effects on the growth and development of intact plants, including axillary bud outgrowth, delay of senescence, and control of nutrient metabolism [3]. Because cytokinin-deficient plants are not available to date, none of the studies conducted to address the physiological role of cytokinins are conclusive. Instead, much of our knowledge of the physiological role of cytokinin is deduced from the effects of cytokinins applied either externally to isolated tissues, cells, and intact plants, or internally through the introduction of bacterial genes encoding cytokinin synthase into plants. Signal transduction pathways initiated by cytokinin have recently begun to be uncovered. This review highlights recent progress in understanding the cytokinin signaling by mainly focusing on recently identified mutants with altered cytokinin responses and genes implicated in cytokinin signaling.

Cytokinin signaling mutants

The *cyr1* mutant of *Arabidopsis* is probably the first identified mutant that shows resistance rather specific to cytokinin [4]. Externally applied auxin, cytokinin, ethylene and abscisic acid retard root elongation, although the biological significance of this observation is unclear. Root elongation of this mutant is resistant to cytokinin but not to other hormones and the plants fail to accumulate anthocyanin in response to cytokinins. The mutation also causes pleiotropic effects, including very limited growth of the shoot, reductions in cotyledon and leaf expansion, and the formation of a single infertile flower. Because expansion of cotyledons and leaves is known to be an effect of cytokinin, reduction in expansion is consistent with what would be expected for cytokinin-insensitive phenotypes. Given that cytokinin is postulated to play a key role in plant development, mutants affected in cytokinin signal transduction may grow poorly and be sterile, like the *cyr1* mutant. Hence, additional cytokinin-signaling mutants could be isolated through extensive genetic screens.

When wild-type *Arabidopsis* seedlings are grown in the presence of low levels of cytokinins in the dark, the seedlings display ethylene responses called the triple response (radial expansion of the hypocotyl, inhibition of hypocotyl and root elongation, and exaggeration of the curvature of the apical hook) [5], because applied cytokinin increases ethylene biosynthesis [5,6••]. These responses were used to isolate mutants that are disrupted in signaling events leading from cytokinin perception to ethylene biosynthesis [6••,7••]. Mutants that do not exhibit the triple response in response to cytokinin, but do exhibit it in response to ethylene were isolated. These fell into five complementation groups, *cin 1–5*. The *cin5* mutation disrupted *ACS5*, a member of the *Arabidopsis* gene family that encodes 1-aminocyclopropane-1-carboxylate synthase (ACC synthase). ACS5 is responsible for the ethylene biosynthesis stimulated by cytokinin in the dark, and appears to be post-transcriptionally regulated by cytokinin. The *cin1* mutant shows cytokinin resistance in multiple assays, including ethylene production, anthocyanin biosynthesis, and shoot formation; however, *cin1* mutant plants are unaffected in leaf senescence (a process delayed by cytokinin application) and in the ability to induce the cytokinin inducible gene, *IBC6* (see below). Thus, *CIN1* may play a role in several but not all of the cytokinin actions. The *cin2* mutation affects ethylene production induced by multiple stimuli. The *cin3* mutation has specific effects on ethylene production induced by cytokinin, but not on other cytokinin responses. *cin4* is allelic to the constitutive photomorphogenic mutants *fus9* and *cop1* and tends to de-etiolate in the dark. These *cin* mutants will contribute to the dissection of the signaling pathways between cytokinin perception and ethylene production.

Arabidopsis mutants with increased sensitivity to cytokinins have also been isolated by means of a tissue culture method. Callus proliferation requires both auxin and cytokinin. Normally, in the presence of auxin, increasing levels of cytokinins are associated with increasing growth and greening of calli. The *ckh1* and *ckh2* mutants display hypersensitivity to cytokinins in these greening and proliferation responses (T Kakimoto, M Kubo,

M Shindo, Abstract 4–35, 8th International Conference on *Arabidopsis* Research, 25–29 June 1997, Madison, Wisconsin). Similar mutants have also been isolated by another group (A Cary, S Howell, Abstract 4-9, 8th International Conference on *Arabidopsis* Research, 25–29 June 1997, Madison, Wisconsin).

Involvement of two-component systems

The two-component system

Until recently, two-component systems were thought to exist only in prokaryotes, however, they are now established that two-component systems exist also in eukaryotes. In plants, signal transduction pathways initiated by ethylene and cytokinin appeared to involve two-component systems; a two-component system typically consists of a sensor histidine kinase (termed the sensor) and a response regulator [8]. Sensors consist of a variable input domain and a conserved histidine kinase (also called the transmitter) domain, and may also have a receiver domain. Response regulators consist of a receiver domain and an output domain. When the input domain of a sensor perceives a signal, the histidine kinase domain is autophosphorylated. The phosphoryl group is ultimately transferred to the receiver domain of the cognate response regulator. In some two-component systems the phosphoryl group is transferred directly from a sensor to a response regulator, whereas in others it is first transferred to another protein containing the histidine-containing phosphotransfer (HPt) domain and then transferred to a response regulator [9,10,11]. Phosphorylation of the receiver domain (of the response regulator) regulates the activity of an attached output domain. In bacteria, many output domains function as transcriptional regulators. There are several response regulators, however, that are entirely composed of a receiver domain (without obvious output domain) but regulate the activity of the target protein (e.g. regulation of flagellar rotation by CheY [12]).

CKI1, a sensor for cytokinin?

Activation T-DNA tagging was used to isolate *Arabidopsis* mutant lines that constitutively exhibit cytokinin responses independently of cytokinin [13]. A large number of calli were transformed with a tagging vector, pPCV1CEn4HPT [14], carrying a tetramer of the cauliflower mosaic virus (CaMV) 35S RNA enhancer, with the expectation that the integrated T-DNA would activate the transcription of the adjacent genes to the integration site, thus creating dominant mutants. Through screening of the transformants, five mutant calli (*cki1-1*, *-2*, *-3* and *-4*, and *cki2*), which exhibited typical cytokinin responses including rapid proliferation, greening, shoot formation and inhibition of root formation in the absence of cytokinin, were isolated. The *CKI1* gene was cloned as the causal gene for the *cki1* phenotype, and its product consists of a putative input domain, a histidine kinase domain and a receiver domain. The *CKI1* gene was tagged 4 times independently (*cki1-1*,*-2*,*-3* and *-4*). Overexpression of the *CKI1* gene induces typical cytokinin responses in the absence of exogenous cytokinin. The *Arabidopsis* ETR1, which also has a histidine kinase domain and a receiver domain [15], was proven to be an ethylene receptor through its ability to bind ethylene [16]. Thus, it is attractive to hypothesize that CKI1 is a cytokinin receptor. Because the *CKI* gene was identified by activation tagging, it is important to know the effects of reduction or elimination of the *CKI1* gene expression. The hunt for the loss-of-function mutants of the *CKI1* gene can be achieved by introducing antisense *CKI1*, or by disrupting the *CKI1* gene. In addition, to test whether the CKI1 protein binds cytokinin will be critically important.

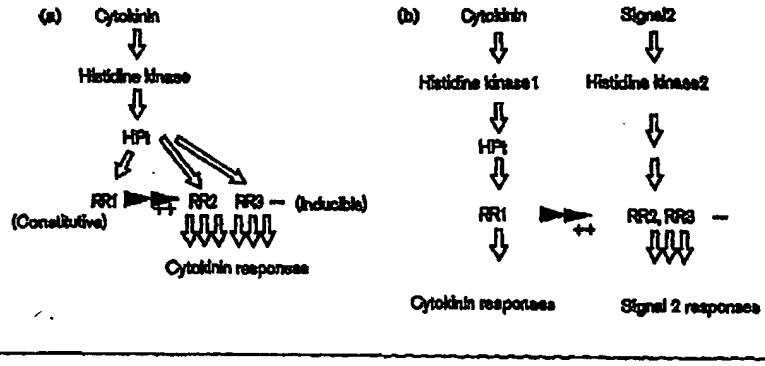
Cytokinin-inducible response regulator

Further evidence implicating the involvement of a two-component system in cytokinin signaling has emerged from recent molecular cloning of genes encoding reponse regulators. A gene (*ZmCip1*) that is inducible by cytokinin was isolated through differential display screening [17••]. Cytokinin increased specifically the ZmCip1 protein level also. *ZmCip1* encodes a product exhibiting similarity to response regulators of two-component systems. The transcript level is increased by physiological levels of cytokinin (as low as 10^{-9} M zeatin), and the increase occurs rapidly and is transient. The increase in the message is insensitive to cycloheximide, an inhibitor of translation, suggesting that the induction does not require *de novo* protein synthesis. In this study, nitrogen-starved maize seedlings were used as material, because high levels of nitrogen nutrients had been reported to increase the endogenous levels of cytokinin [18]. Indeed, nitrogen re-supply increased the levels of both the endogenous cytokinin and the *ZmCip1* mRNA transcript [17••]. In addition to the intrinsic importance of the cloned gene that insists the involvement of a response regulator in cytokinin signaling, the results further support the idea that cytokinin transmits the information regarding the nitrogen level in soil from the root to the shoot.

Another group has simultaneously carried out similar screening for cytokinin-inducible genes in *Arabidopsis* [19••]. In an attempt to deplete endogenous cytokinin in the starting material, roots were removed from etiolated seedlings, because the roots are believed to be the major site of cytokinin synthesis, and then the remaining aerial portions of the seedlings were incubated in liquid medium, before treatment with cytokinin. One of the isolated genes, *IBC6*, encoded a product with sequence similarity to response regulators. IBC6 is highly homologous to ZmCip1, with about 60% amino acid identity over the response regulator domain. *IBC7* was cloned as a gene homologous to *IBC6*, and is also inducible by cytokinin. The transcript level of *IBC6* is significantly and rapidly increased by cytokinin, slightly increased by abscisic acid after a longer lag, but not altered by auxin, gibberellin, ethylene or light. The induction of *IBC6* and *IBC7* by cytokinin does not require *de novo* protein synthesis.

An expressed sequence tag (EST) search was also performed to isolate genes encoding homologs of response regulators, and five cDNAs (*ARR3–7*) were cloned fro

Figure 2



Two possible models for cytokinin signaling. These models are formed on the assumptions that cytokinin is perceived by a two-component system, and that a response regulator is downstream of a sensor. (a) In this model, a cytokinin signal up-regulates response regulators RR2 and RR3 (and more RRs), which function downstream of a cytokinin receptor. (b) In this model, cytokinin-inducible response regulators are under a signal other than cytokinin. Through the cross-talk presented here, cytokinin modulates another signaling pathway. RR1, constitutively expressed response regulator; RR2 and RR3, response regulators up-regulated by cytokinin. In these models, RR1 regulates RR2 and RR3, directly or indirectly. HPt, mediator of phosphotransfer, may or may not be involved. Transcriptional activation is indicated by double arrowheads.

identity to the *Dictyostelium* cAMP receptor, a heterotrimeric G-protein coupled receptor. Heterotrimeric G-proteins are prevalent in animals and fungi, and relay signals from seven-transmembrane receptors. Transformants expressing the antisense *GCR1* gene are less responsive to cytokinin compared to wild-type in root elongation assay. If the gene product is indeed a G-protein coupled receptor, an important issue is the identification of its ligand.

Conclusions

Our understanding of cytokinin signaling has been considerably enhanced over the past couple of years. Lines of evidence suggest that a two-component system(s) is central to the cytokinin signaling, although this notion remains to be verified. Is the CKI1 protein indeed a cytokinin receptor? What is the role of the cytokinin-inducible response regulators? Are the response regulators downstream of the CKI1 protein? Biochemical and molecular genetic approaches will answer these questions. Because genes for several response regulators are primary-response genes to cytokinin, analysis of their promoter sequences and identification of trans-acting factors that bind to the promoters, will also be important. The GCR1 seven-transmembrane protein is also a candidate for a cytokinin receptor. To test whether CKI1 and GCR1 bind cytokinin is critically important. The next five years will be an important time for research on cytokinin signaling, and the time may soon come when we can build a framework model for the signal transduction pathway. Finally, further exciting issues to be addressed are; how cytokinins are synthesized and what cytokinin is really doing in plants.

Acknowledgements

The author is very grateful to Keiko Torii for valuable comments on this paper, Joseph Kieber, Richard Hooley, Takeshi Mizuno, Hitoshi Sakakibara, Mitsutaka Taniguchi and Kazuo Shinozaki for providing preprints for the review. Work by the author's group was supported by the Ministry of Education, Science, and Culture of Japan (Grant Nos. 09274217, 09274102 and 06278103), the Science and Technology Agency (Special Coordination Funds for Promoting Science and Technology), the Sumitomo Foundation and the Nissan Foundation.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Miller CO, Skoog F, von Saltza HM, Okumura FS, Strong FM: Kinetin: Structure and synthesis of kinetin. *J Am Chem Soc* 1955, 77:2662-2663.

2. Skoog F, Miller CO: Chemical regulation of growth and organ formation in plant tissues cultured *in vitro*. *Symp Soc Exp Biol* 1957, 11:118-131.

3. Mok DWS, Mok MC: *Cytokinins: Chemistry, Activity and Function*. Boca Raton, Florida: CRC Press; 1994.

4. Deikman J, Ulrich M: A novel cytokinin-resistant mutant of *Arabidopsis* with abbreviated shoot development. *Planta* 1995, 195:440-449.

5. Cary AJ, Liu W, Howell SH: Cytokinin action is coupled to ethylene in its effects on the inhibition of root and hypocotyl elongation in *Arabidopsis thaliana* seedlings. *Plant Physiol* 1995, 107:1075-1082.

6. •• Vogel JP, Woeste KE, Theologis A, Kieber JJ: Recessive and dominant mutations in the ethylene biosynthetic gene *ACS5* of *Arabidopsis* confer cytokinin insensitivity and ethylene overproduction, respectively. *Proc Natl Acad Sci USA* 1998, 95:4766-4771.

Interactions between different plant hormones are important for plant development. Cytokinin is known to induce ethylene. *cin5* is one of the mutants isolated by Kieber's group [7••], and does not respond to low levels cytokinin by producing ethylene. The *cin5* mutation disrupts the *ACS5* gene that encodes one of the 1-aminocyclopropane-1-carboxylate synthases.

7. •• Vogel JP, Schuerman P, Woeste K, Brandstatter I, Kieber JJ: Isolation and characterization of *Arabidopsis* mutants defective in the induction of ethylene biosynthesis by cytokinin. *Genetics* 1998, 149:417-427.

Large scale screening was carried out to identify mutants in which ethylene biosynthesis is not induced by low levels of cytokinin. The identified mutations represent five genetic loci, and disrupted different steps leading from cytokinin perception to ethylene production.

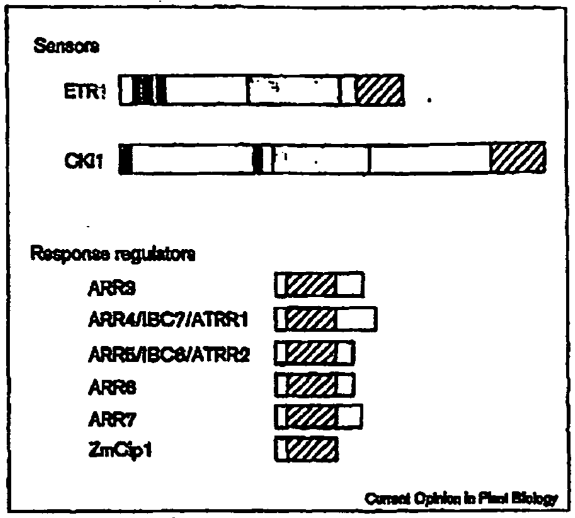
Arabidopsis. ARR proteins appeared to have the ability to accept the phosphoryl group from a protein containing the histidine-containing phosphotransfer (HPt) domain in *E. coli*., and furthermore they were shown to accept it *in vitro*. Genes encoding products with characteristic HPt sequences have also been cloned from *Arabidopsis* [20••]. Somewhat surprisingly, the mRNA transcripts for all five genes (*ARR3–7*) were increased by cytokinin. Accumulation of the mRNA for *ARR4*, a gene chosen for detailed analysis, was specifically increased by cytokinin [21••]. Additional cDNA clones encoding response regulator homologs (*ATRR1–4*) have been cloned in a different laboratory [22•]. *ATRR1* and *ATRR2* were induced by low temperature.

In the studies cited above, the same genes were cloned independently. *ARR4* is identical with *ATRR1* and *IBC7*, except for one amino acid change in *IBC7*; *ARR5* is identical with *ATRR2* and *IBC6*, except for two amino acid changes in *IBC7*. The difference in ecotypes of *Arabidopsis* is probably responsible for these amino acid differences (*ARRs* and *ATRRs* were cloned from the Columbia ecotype and *IBCs* were cloned from the Wassilewskija ecotype). Because the receiver domains of *ARR3–7* are highly homologous to each other, they may represent a subfamily of a possible response regulator family and may function downstream of the same input signal, possibly cytokinin. Considering the existence of multiple genes for putative sensor histidine kinases in *Arabidopsis*, including *CKI1*, *CKI2* (the gene tagged in the *cki2* mutant; T Kakimoto, unpublished data), genes for ethylene receptors ETR1 and ERS, as well as several putative genes encoding sensor histidine kinase homologs identified in the *Arabidopsis* Genome Project, there may be unidentified groups of response regulators functioning under different signals.

Models for cytokinin signal transduction mediated by two-component systems

Each of the response regulators cited above has variable amino-terminal and carboxy-terminal extensions attached to the core receiver domain (Figure 1). Carboxy-terminal extensions of ARR3 and ARR4/IBC7/ATRR1 contain an acidic region and a proline rich region. Although no DNA binding regions are found in these proteins, acidic and proline rich regions possibly function as transcriptional activators. In bacteria, a number of response regulators are transcriptionally regulated by a positive feed-back loop to stimulate their own proteins [23,24,25]. For example, transcription of *Spo0A*, which encodes a response regulator in *Bacillus subtilis*, is regulated by the phosphorylated Spo0A protein [23,24]. Similarly, cytokinin inducible response regulators may locate downstream of a sensor kinase(s) that senses cytokinin, and their protein level may be regulated by cytokinin. A good candidate for the censor is the CKI1 protein. If the protein levels of these response regulators were regulated by their own proteins, however, induction of their mRNA accumulation would be sensitive to inhibitors of translation. Because induction of these genes by cytokinin are resistant to cycloheximide [17••,19••], these response regulators may be regulated by another protein whose activity is regulated by cytokinin (Figure 2a). Alternatively, the cytokinin-inducible response-regulators may act downstream of a sensor for another signal, and up-regulation by cytokinin might modulate the responsiveness to that signal (Figure 2b).

Figure 1



Two-component sensors and response regulators in plants. Shaded boxes represent histidine kinase domains, Hatched boxes represent receiver domains, and closed boxes represent transmembrane segments. ETR1 is an ethylene receptor. CKI1 is possibly a cytokinin receptor. Genes encoding response regulators depicted in this figure are all inducible by cytokinin.

Other genes implicated in cytokinin signaling

Although recent cloning of *CKI1* and response regulators emphasizes the involvement of a two component system in cytokinin signaling, the following two reports implicate a presence of additional signaling mechanisms in cytokinin perception.

The first report describes the tobacco mutant lines, *cyi1–4*, which were isolated through activation tagging and proliferate even in the absence of both auxin and cytokinin in tissue culture. All *cyi* plants displayed common phenotypes including reduced apical dominance, poorly developed roots, delayed growth, and male- and female-sterility. The *CYI1* cDNA contains an open reading frame of 22 amino acids, and over-expression of the open reading frame causes auxin- and cytokinin-independent growth [26•]. It should be noted, however, that some of these data are under reassessment.

The second example is the *Arabidopsis* gene *GCR1*, which encodes a product with a putative seven transmembrane receptors [27••]. GCR1 protein exhibits 18–23% amino acid

8. Hoch JA, Silhavy TJ: *Two-Component Signal Transduction.* Washington DC: ASM Press: 1995.

9. Popas F, Wurgler-Murphy M, Maeda T, Witten EA, Thai TC, Saito H: Yeast hog1 MAP kinase cascade is regulated by a multistep phosphorelay mechanism in the SLN1-YPD1-SSK1 'two-component' osmosensor. *Cell* 1996, 86:865-875.

10. Appleby JL, Parkinson JS, Bourret RB: Signal transduction via the multi-step phosphorelay: not necessarily a road less traveled. *Cell* 1996, 86:845-848.

11. Kato M, Mizuno T, Shimizu T, Hakoshima T: Insights into multistep phosphorelay from the crystal structure of the c-terminal HPt domain of ArcB. *Cell* 1997, 88:717-723.

12. Welch MK, Oosawa K, Aizawa S-I, Eisenbach M: Phosphorylation-dependent binding of a signal molecule to the flagellar switch of bacteria. *Proc Natl Acad Sci USA* 1993, 90:8787-8793.

13. Kakimoto T: CKI1, a histidine kinase homolog implicated in cytokinin signal transduction. *Science* 1996, 274:982-985.

14. Hayashi H, Czaja I, Lubenow H, Schell J, Walden R: Activation of a plant gene by T-DNA tagging: auxin-independent growth *in vitro*. *Science* 1992, 258:1350-1353.

15. Chang C, Kwok SF, Bleecker AB, Meyerowitz EM: *Arabidopsis* ethylene-response gene *ETR1*: similarity of product to two-component regulators. *Science* 1993, 262:539-544.

16. Schaller GE, Bleecker AB: Ethylene-binding sites generated in yeast expressing the *Arabidopsis ETR1* gene. *Science* 1995, 270:1809-1811.

17. •• Sakakibara H, Suzuki M, Takei K, Deji A, Taniguchi M, Sugiyama T: A response regulator homolog possibly involved in nitrogen signal transduction mediated by cytokinin in maize. *Plant J*, 1998, in press.

Through differential display screening, a gene that was clearly increased by cytokinin in nitrogen-starved maize was identified and named *ZmCip1*. The cytokinin-induced expression of *ZmCip1* was not inhibited by cycloheximide, suggesting that the induction does not require *de novo* protein synthesis. *ZmCip1* encodes a homolog of response regulators of the two-component systems. This result, together with the fact that over-expression of the *CKI1* histidine kinase gene induces typical cytokinin responses in the absence of cytokinin [13], suggests that a two-component system(s) is involved in cytokinin signal transduction. Nitrogen re-supplying increased the cytokinin level and also increased the message for *ZmCip1*. These results suggest that when a root senses an increase in the nitrogen level in the soil, it increases the cytokinin level. Increased cytokinins may be transported to the shoot and induce the expression of *ZmCip1*.

18. Samuelson ME, Eliasson L, Larsson C-M: Nitrate-regulated growth and cytokinin responses in seminal roots of barley. *Plant Physiol* 1992, 98:309-315.

19. •• Brandstatter I, Kieber J: Two genes with similarity to bacterial response regulators are rapidly and specifically induced by cytokinin in *Arabidopsis*. *Plant Cell* 1998, in press.

This work and similar work by Sakakibara *et al.* [17••] were carried out at the same time. Through differential display screening, genes (*IBC6* and *IBC7*) induced specifically by cytokinins were cloned from *Arabidopsis*, and were shown to encode response regulator homologs. Like the *ZmCip1* induction [17••], the cytokinin-induction of the expression of *IBC6* and *IBC7* does not require *de novo* protein synthesis.

20. •• Imamura A, Hanaki N, Umeda H, Nakamura A, Suzuki T, Ueguchi C, Mizuno T: Response regulators implicated in His-to-Asp phosphotransfer signaling in *Arabidopsis*. *Proc Natl Acad Sci USA* 1998, 95: 2691-2696.

Five cDNAs (*ARR3–7*) encoding response regulator homologs were isolated from *Arabidopsis* through an EST search and library screening. ARR proteins appeared to have the ability to accept the phosphoryl group from a protein containing the HPt domain in *E.coli*, and furthermore they were shown to accept it *in vitro*. These results suggest that the isolated genes encode functional response regulators.

21. •• Taniguchi M, Kiba T, Sakakibara H, Ueguch C, Mizuno T, Sugiyama T: Expression of *Arabidopsis* response regulator homologs is induced by cytokinins and nitrate. *FEBS Lett* 1998, in press.

Messages for *ARR3–7* [20••] were increased by cytokinin, and were also increased by re-supplying of nitrogen to nitrogen-starved *Arabidopsis*. This report and reference [17••] suggest that cytokinin may function as a messenger from the root to the shoot, which may transmit information of the nutrient status in the soil.

22. • Urao T, Yakubov B, Yamaguchi-Shinozaki K, Shinozaki K: Stress responsive expression of genes for two-component response regulator-like proteins in *Arabidopsis thaliana*. *FEBS Lett* 1998, in press.

Four cDNAs for genes (*ATRR1–4*) encoding response regulator homologs were cloned by utilizing the EST database and low-stringency hybridization. *ATRR1* and *ATRR2* were induced by low temperature and other stresses. *ATRR1* and *ATRR2* were reported to be induced by cytokinin [17••,18].

23. Strauch MA, Trach KA, Day J, Hoch JA: Spo0A activates and represses its own synthesis by binding at its dual promoters. *Biochemie* 74:619-626.

24. Strauch M, Wu JJ, Jones RH, Hoch JA: A positive feed-back loop controls transcription of the *spo0F* gene, a component of the sporulation phosphorelay in *Bacillus subtilis*. *Mol Microbiol* 1992, 7:967-974.

25. Stachel SE, Zambryski PC: virA and virG control the plant-induced activation of the T-DNA transfer process of *A. tumefaciens*. *Cell* 1986, 46:325-333.

26. • Mildasheviche E, Czaja I, Cordeiro A, Prinsen E, Schell J, Walden R: T-DNA tagging reveals a novel cDNA triggering cytokinin- and auxin-independent protoplast division. *Plant J* 1997,12:489-498.

Activation tagging was employed to isolate tobacco mutants that proliferate in the absence of cytokinin. Cells of the isolated four mutants (*cyl1–4*) were able to proliferate in the absence of both auxin and cytokinin. The *CYl1* cDNA contains an open reading frame of 22 amino acids, and over-expression of the open reading frame causes auxin- and cytokinin- independent growth. It should be noted, however, that some of these data are under reassessment.

27. •• Plakidou-Dymock S, Dymock D, Hooley R: A higher plant seven transmembrane receptor that influences sensitivity to cytokinins. *Curr Biol* 1998, 8:315-324.

This is the first report of a gene (*GCR1*) encoding a putative seven transmembrane protein in plants. The product exhibits homology to the *Dictyostelium* cAMP receptor, which is a heterotrimeric G-protein coupled receptor. *Arabidopsis* lines transformed with antisense *GCR1* are less sensitive to cytokinin in a root-elongation assay.

An *Arabidopsis* histidine kinase is essential for megagametogenesis

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Cytokinin-Independent 1 (CKI1) belongs to a group of putative plant histidine kinases whose members do not appear to act as ethylene receptors. The deduced protein structure, combined with the observation that *Arabidopsis* callus cultures overexpressing CKI1 exhibit a "cytokinin-independent" cell division and greening phenotype, led to the hypothesis that CKI1 is involved in cytokinin signaling, perhaps acting as a cytokinin receptor. To test the function of CKI1, we used a reverse-genetic approach to identify plants carrying T-DNA insertions in *CKI1*. Two independent alleles were identified, which produce the same developmental phenotype. Analyses of populations segregating for the *cki1-5* or *cki1-6* T-DNA insertion alleles failed to reveal any homozygous *cki1* plants, indicating that the homozygous mutant condition was lethal. Based on segregation distortion, transmission studies, a microscopy-based examination of developing female gametophytes, and mRNA expression data, we suggest that CKI1 function is required for megagametophyte development. Our work with *CKI1* mutants indicates that signal transduction by means of a His/Asp phosphorelay system may play an important and previously unsuspected role in female gametophyte development in *Arabidopsis*.

The histidine to aspartate (His/Asp) phosphorelay is a well characterized prokaryotic signal-transduction pathway. Bacteria use this system to respond to a wide range of changes in their environment, including fluctuations in osmolarity, nutrient availability, and oxygen levels (1). Homologues of the three key proteins in a His/Asp phosphorelay, namely the histidine kinases, histidine-containing phosphotransmitters (HPts), and response regulators, have all been identified in *Arabidopsis* (for review see ref. 2) Aside from higher plants, the only eukaryotes in which these proteins have been found are yeast (3), *Dictyostelium* (4), and *Neurospora* (5).

The sequencing of the *Arabidopsis* genome has revealed 11 genes encoding histidine kinase-like proteins. Five of these proteins have been characterized as ethylene receptors (ETR1, ETR2, ERS1, ERS2, and EIN4) (6), and three are cytokinin receptors (CRE1 and its homologues AHK2 and AHK3) (7, 8). Indirect evidence implicates the remaining three histidine kinases in osmosensing (ATHK1) (9), as well as in cytokinin signaling (CKI1 and CKI2) (10).

Although plant responses to cytokinins have been studied since the discovery of kinetin in 1956 (11), the first and long-sought-after cytokinin receptor (CRE1) was identified only little more than 1 year ago (7). This major breakthrough in the field of cytokinin signaling occurred amid a renewed interest in identifying proteins that may participate in the cytokinin-signaling pathway (8, 12–15). It has now been established that an His/Asp phosphorelay plays a role in cytokinin signaling (for model see ref. 16), although the particular histidine kinases, response regulators, and HPts that mediate specific cytokinin responses have not yet been identified. The growing body of research linking *Arabidopsis* His/Asp phosphorelay signaling proteins to cytokinin signal transduction was initiated by Kakimoto's discovery of CKI1 (Cytokinin-Independent 1) (10).

The first nonethylene receptor histidine kinase described was CKI1. Overexpression of *CKI1* was found to confer cytokinin-inducible responses on *Arabidopsis* callus tissue in the absence of applied cytokinin (10). Thus, CKI1 was initially implicated in hormone signaling. To explore the function of CKI1, we used a reverse-genetic approach to identify plants containing null alleles of *CKI1*. A genome-wide method to obtain null alleles by means of T-DNA insertions is well established in *Arabidopsis thaliana* (17). Here, we describe two independent T-DNA insertion alleles of *CKI1* that have the same effect on plant development. Based on segregation distortion, transmission studies, a microscopy-based analysis of mutant ovules, and mRNA expression data, we have identified a role for CKI1 in megagametogenesis in *A. thaliana*.

Materials and Methods

Plant Materials and Growth Conditions. Seeds of *A. thaliana*, ecotypes Wassilewskija (Ws) and Landsberg *erecta* (*Ler*), were germinated on plates containing half-strength Murashige and Skoog salts (18), 1% (wt/vol) sucrose, and 0.8% (wt/vol) washed agar (MS plates). Seedlings were transferred to soil after ≈8 days. The plants were then subjected to the following growth conditions: either 21°C under constant light, or 22°C with a light regime of 16 h light/8 h dark. Both T-DNA insertion lines studied were backcrossed at least twice before genetic transmission studies, CAPS (cleaved amplified polymorphic sequences) analysis, and microscopy. For transmission studies, reciprocal crosses were performed between mutant and wild-type plants. Seeds were harvested from individual siliques and germinated on MS plates containing 50 μg/ml kanamycin. The T-DNA construct used (pD991) carries a selectable marker gene that renders transgenic plants resistant to kanamycin (Kan^R).

Screening for T-DNA Insertion Lines. T-DNA insertion alleles were obtained by screening the α population of 60,480 independent T-DNA insertion lines, provided by the *Arabidopsis* Knockout Facility at the University of Wisconsin (www.biotech.wisc.edu/Arabidopsis/). Details of the screening procedure are described in ref. 17. The *CKI1* gene-specific primers used were CRFF (5′-CGCAGCCAAACTATTATTTTACCACAGAC-3′) and CR1M1 (5′-ATCGAGCCATTGGAGATGAAGAAA-GAATC-3′). The T-DNA-specific primers used were left border (5′-CATTTTATAATAACGCTGCGGACATCTAC-3′) and right border (5′-TGGGAAAACCTGGCGTTACCCAACT-TAAT-3′). The location of the T-DNA insert relative to the *CKI1* genomic sequence was determined by sequencing PCR products containing the T-DNA/plant genomic DNA junction. The genotype of individual plants grown on MS plates was determined by two PCRs. One PCR contained both gene-

Abbreviations: CAPS, cleaved amplified polymorphic sequences; CKI1, Cytokinin-Independent 1; CLSM, confocal laser scanning microscopy; FGn, female gametophyte stage *n*; HPt, histidine-containing phosphotransmitter; Kan^R, kanamycin resistant; Kan^S, kanamycin sensitive.

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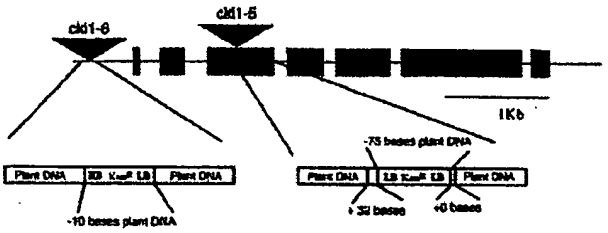

Fig. 1. Description of the *CKI1* T-DNA insertion alleles. Black boxes represent exons and triangles represent T-DNA integration sites. The T-DNA insertion allele *cki1-6* contains an insert 587 bases upstream of the ATG, as well as a deletion of 10 bases at the integration site. The T-DNA insertion allele *cki1-5* contains an insert 257 bases into the third exon, as well as a deletion of 75 bases and an addition of 40 bases of unknown origin at the integration site. LB and RB, left and right borders of the T-DNA, respectively; Kan^R, kanamycin resistance gene (neomycin phosphotransferase).

specific primers, identifying the presence of a wild-type allele. The other PCR contained the appropriate combination of a gene-specific and a T-DNA-specific primer, identifying the presence of a mutant allele.

CAPS Analysis. Two sets of primers were selected from the CAPS marker table on the Arabidopsis Information Resource (TAIR) web site (www.arabidopsis.org/aboutcaps.html). Primers were selected based on their ability to yield a polymorphism between the ecotypes Ws and *Ler* after PCR and digestion of the amplified product with the appropriate restriction enzyme. The primers chosen for CAPS analysis were 5′-GGGATTTGATGAAGGAGAAC-3′ and 5′-ATTCCTTGGTCTCCATCATC-3′, corresponding to the marker GPA1a on chromosome 2, as well as 5′-ACTCCTTTGTCATCTCCCGAATC-3′ and 5′-CCAACAACATGCATGATAGTTCAG-3′, corresponding to the marker 17D8LE on chromosome 3. Genomic DNA was prepared from the leaf tissue of Kan^R progeny resulting from crosses between wild-type (*CKI1/CKI1*) *Ler* and heterozygous (*cki1/CKI1*) Ws plants. DNA isolation, PCR, and restriction digests were carried out as described (19, 20). The ecotype (Ws/Ws, *Ler*/*Ler*, or Ws/*Ler*) of Kan^R plants was determined by the size of digested PCR products visualized on 1% (wt/vol) agarose gels.

Confocal Laser Scanning Microscopy (CLSM). Tissue preparation, microscopy, image capture, and figure preparation were performed as described (21, 22).

***In Situ* Expression Analysis.** Nonradioactive *in situ* hybridization experiments were carried out as described (23) with the following modification: tissue was fixed and embedded as described (24). *CKI1* antisense probes were prepared to exons 1–4 and exon 6 of *CKI1*. A *FILAMENTOUS FLOWER* [member of the *YABBY* gene family (25)] probe was used as a positive control.

Molecular Complementation. After amplification of Ws genomic DNA with PCR primers 5′-AATAATTGGGAAAACATGTGATAAAAGTCTGA-3′ and 5′-GGCGCGCCCACTGGTTTCATTTGCCTACAT-3′, and subsequent restriction digests with *Pac*I and *Asc*I (NEB, Beverly, MA), an ≈6.5-kb genomic fragment containing the *CKI1* gene, 1,300 bases upstream of the start codon, and 185 bases downstream of the stop codon, was ligated into pCAMBIA3300S, a spectinomycin-resistant derivative of pCAMBIA3300 (26). Plasmids containing the *CKI1* gene were introduced by electroporation into *Agrobacterium tumefaciens*, and used to transform *cki1/CKI1 Arabidopsis* plants by means of the floral dip method (27). Transformed plants were selected on MS plates containing 25 μg/ml ammonium glufosinate (Sigma–Aldrich), the active ingredient of the herbicide BASTA. The T_2 generation of transformed plants (the plant that was dipped was designated generation T_0) was analyzed for complementation of the *cki1* phenotype by kanamycin segregation as described. Complementation was confirmed by three PCRs. The first PCR contained the gene-specific primer 5′-AATAGGCTTTCGACCGGTACGCACTGACT-3′ and the T-DNA-specific primer 5′-TTTCTCCATATTGACCATCATACTCATTG-3′, identifying the presence of the disrupted *cki1* allele. The second PCR contained the gene-specific primer 5′-CCTATGGAGATGCGTAAGTCGGTATTTGA-3′ and T-DNA-specific primer 5′-GTCATGCCAGTTCCCGTG-3′, identifying the presence of the transgene. The third PCR contained the gene-specific primers 5′-GAACGGGTCAGAACATTAAAACATACATT-3′ and 5′-TCTTCCCGCTTTCGATTTTGCTCAT-3′, identifying the presence of the endogenous wild-type *CKI1* allele. A complemented plant was defined as a plant lacking the endogenous wild-type allele of *CKI1*, yet viable because of the presence of the transgene.

Results

We identified *Arabidopsis* plants containing two independent T-DNA insertion alleles of *CKI1*. *cki1-5* contains an insert within the third exon, and *cki1-6* contains an insert 587 bases upstream of the translation start site (Fig. 1). For each allele, both T-DNA/plant genomic DNA junctions were sequenced to rule out the possibility of chromosomal translocations or large deletions (28). Analysis of the progeny resulting from self-

Table 1. Genetic analysis of insertion alleles *cki1-5* and *cki1-6*

Cross performed		Resultant progeny		
Female parent*	Male parent	*cki1/CKI1*:*CKI1/CKI1*	Kan^R:Kan^S	T-DNA transmission†, %
cki1-5/CKI1	*cki1-5/CKI1*	162:178	ND	47.6
cki1-6/CKI1	*cki1-6/CKI1*	145:144	ND	50.2
cki1-5/CKI1	*CKI1/CKI1*	ND	27:1089	2.4
cki1-5/CKI1	*CKI1/CKI1*‡	ND	5:160	0.0§
cki1-6/CKI1	*CKI1/CKI1*	ND	21:210	9.1
cki1-6/CKI1	*CKI1/CKI1*‡	ND	12:173	0.0§
CKI1/CKI1	*cki1-5/CKI1*	ND	589:549	51.3
CKI1/CKI1	*cki1-6/CKI1*	ND	143:160	47.2

ND, not determined.
*Ecotype Wassilewskija.
†Percent T-DNA transmission was calculated as 100 × Kan^R/(Kan^R + Kan^S).
‡Ecotype Landsberg *erecta*.
§Percent T-DNA transmission was determined by a CAPS analysis.

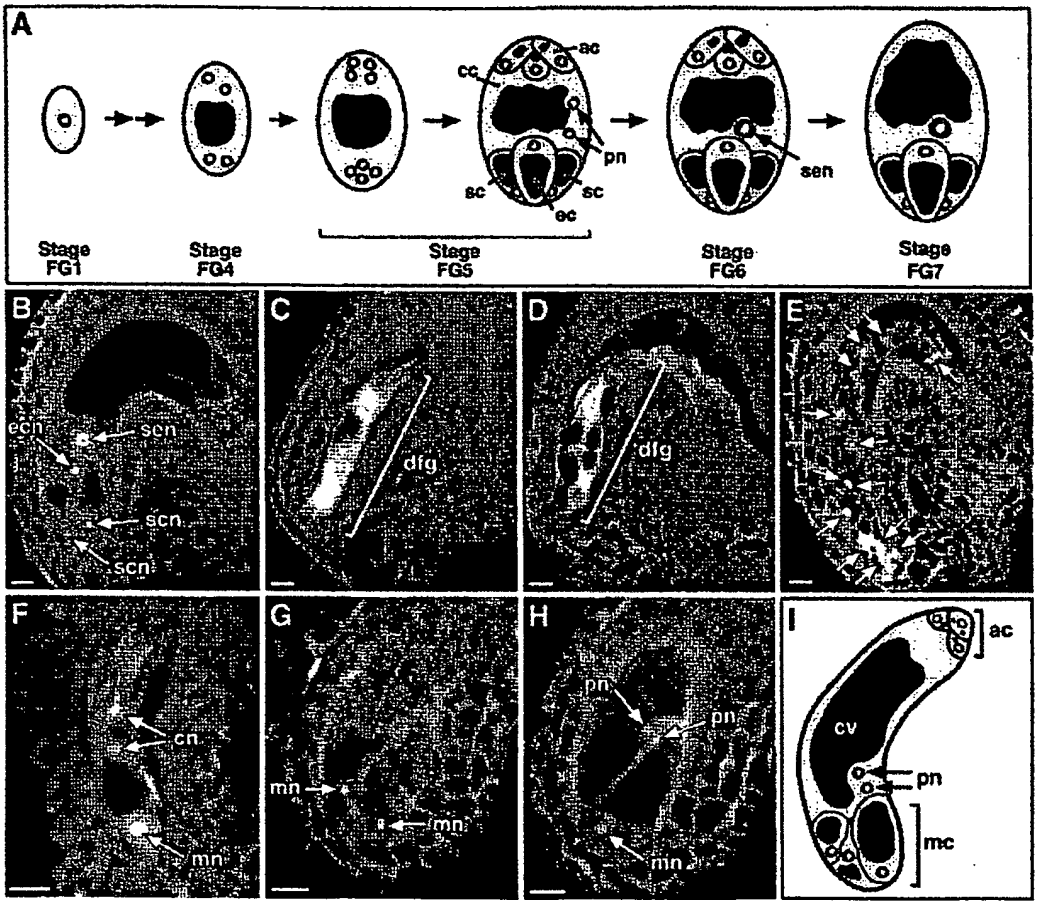

Fig. 2. Phenotypic analysis of *cki1-5* female gametophytes. (*A*) Depiction of wild-type female gametophyte development. Megagametogenesis has been described and divided into seven stages (21). Before megagametogenesis, a diploid megaspore mother cell undergoes meiosis and produces four haploid megaspores, three of which degenerate. The surviving megaspore defines stage FG1. The megaspore then undergoes three rounds of mitosis without cytokinesis, giving rise to an eight-nucleate cell (early stage FG5). Immediately following the third mitosis, cell walls form and partition the nuclei into cellular compartments (late stage FG5). The central cell inherits two nuclei, called the polar nuclei, which fuse to form a homodiploid nucleus (stage FG6). Finally, the antipodal cells degenerate. The mature female gametophyte (stage FG7) consists of one central cell, one egg cell, and two synergid cells (22). (*B–E*) CLSM images of female gametophytes at the terminal developmental stage (stage FG7). (*B*) A wild-type *Arabidopsis* female gametophyte. This image is a projection of two 1.0-μm slices. (*C*) A degenerated *cki1-5* female gametophyte. This image is a projection of five 1.5-μm optical sections. (*D*) A partially degenerated *cki1-5* female gametophyte. This image is a single 1.0-μm optical section. (*E*) A *cki1-5* female gametophyte that contains an excessive number of nuclei (indicated by arrows). This image is a single 1.5-μm optical section. (*F*) CLSM image of a *cki1-5* female gametophyte from an ovary at stage FG4 (i.e., the ovary contains wild-type female gametophytes at stage FG4). This image is a projection of two 1.5-μm optical sections and shows two degenerating chalazal nuclei and a single intact micropylar nucleus. A second intact micropylar nucleus was present but not projected in this image. (*G* and *H*) CLSM images from the same *cki1-5* female gametophyte from an ovary at stage FG5/6 (i.e., the ovary contains wild-type female gametophytes at stage FG5/6). Because of the abnormal cell morphologies, distinct cellular identities could not be assigned for the cells at the micropylar end. The nuclei are thus labeled as micropylar nuclei (mn). The presumed polar nuclei are unfused. Three presumed antipodal cells were observed at the chalazal end but are not projected in these images. Both images are separate projections of two 1.0-μm optical sections each. (*I*) Depiction of a *cki1-5* female gametophyte at the FG5/6 stage illustrating misshapen vacuoles and the irregular placement of micropylar nuclei. All female gametophytes are oriented with their chalazal poles up and their micropylar poles down. ac, antipodal cells; cc, central cell; cn, chalazal nucleus; cv, central cell vacuole; dfg, degenerated female gametophyte; ec, egg cell; ecn, egg cell nucleus; mc, micropylar cells; mn, micropylar nucleus; pn, polar nucleus; sc, synergid cell; scn; synergid cell nucleus; sen, secondary nucleus. (Bar, 10 μm.)

fertilization of plants heterozygous for the *cki1-5* or *cki1-6* alleles did not yield a plant homozygous for the insertion in either allele. This observation indicates that the homozygous condition is lethal for some aspect of plant growth and development. Furthermore, the percent transmission of the mutant allele following the self-fertilization of plants heterozygous for the *cki1-5* or *cki1-6* alleles was ≈50%, which is consistent with a gametophytic defect (ref. 29; Table 1). Results were the same whether the plant genotypes were determined by using PCR or by kanamycin selection (data not shown).

To determine whether T-DNA transmission through the male and/or female gametes was reduced, reciprocal crosses were performed between *cki1-5/CKI1* or *cki1-6/CKI1* plants and wild-type plants. Our results show that mutant and wild-type alleles are transmitted equally well although the pollen (Table 1), but the transmission of mutant alleles through the female gametophyte is severely reduced relative to the wild-type allele (Table 1).

To determine whether transmission through the female gametophyte is completely eliminated, wild-type plants of the ecotype L*er* were used as pollen donors in crosses with *cki1-5/CKI1* or *cki1-6/CKI1* plants (ecotype Ws), and a CAPS analysis was performed on all Kan^R progeny. This procedure allowed

Table 2. Summary of CLSM analysis of the *cki1-5* and *cki1-6* mutants

Genotype	FG stage	No. of pistils analyzed	No. of normal FGs observed	No. of abnormal FGs observed	Abnormal, %
cki1-5/CKI1	FG0–FG3	7	69	0	0
	FG4	8	58	6	9
	FG5/6	13	74	73	50
	FG7	32	173	204	54
cki1-6/CKI1	FG7	19	89	117	56

FG, female gametophyte.

us to distinguish between Kan^R progeny that resulted from a successful cross, and those that were the result of self-fertilization because of imperfect emasculation. Our results indicated that *cki1* T-DNA insertion alleles were never transmitted through the female gametophyte (Table 1), yielding 100% penetrance for this aspect of the mutant phenotype.

We noticed reduced seed set, in the form of empty spaces, within siliques of self-fertilized flowers from *cki1-5/CKI1* and *cki1-6/CKI1* plants (data not shown). Combined with the segregation distortion and transmission studies, this semisterile phenotype supported the idea that disrupting *CKI1* results in a defect in female gametophyte development (29). To determine the nature of the defect in a *cki1* female gametophyte, we analyzed mutant female gametophytes by using confocal laser scanning microscopy (CLSM). In this procedure, nucleoli appear white, cytoplasm appears gray, and vacuoles appear black (Fig. 2*B*) (21).

We first determined the terminal phenotypes of the *cki1* mutants by examining the female gametophytes after allowing megagametogenesis within the ovaries of heterozygous plants to progress to the terminal developmental stage, female gametophyte stage 7 (FG7) (21). Both mutants had morphologically abnormal female gametophytes at stage FG7, indicating that the *cki1-5* and *cki1-6* mutations similarly affect megagametogenesis. With both mutants, approximately half of the female gametophytes we observed exhibited mutant phenotypes (Table 2). This proportion is consistent with the observed 100% penetrance of *cki1-5* and *cki1-6* mutations in the female gametophyte (Table 1).

Embryo sacs of *cki1-5* and *cki1-6* mutants had similar abnormal phenotypes. In both cases, mutant female gametophytes fell into two phenotypic categories. In the first and most frequent (153/204 for *cki1-5* and 114/117 for *cki1-6*) category, female gametophytes appeared to be in the process of degeneration. The defects ranged from partial (Fig. 2*D*) to complete degeneration (Fig. 2*C*). The embryo sac cavity of partially degenerated female gametophytes was collapsed, yet displayed evidence of cellularization (Fig. 2*D*). The embryo sac cavity of completely degenerated female gametophytes was collapsed and filled with brightly fluorescent material (Fig. 2*C*). In the second phenotypic category (51/204 for *cki1-5* and 3/117 for *cki1-6*), the embryo sac cavity was filled with a matrix of cytoplasmic strands connecting many small vacuoles (>100), as well as a greater than normal number of nuclei (≥16) (Fig. 2*E*).

To determine the developmental stage at which megagametogenesis in the mutants first departs from wild type, we analyzed megagametogenesis at all developmental stages in *cki1-5/CKI1* ovaries. Wild-type female gametophyte development is shown in Fig. 2*A*. As summarized in Table 2, abnormal female gametophytes were not observed during the one-nucleate (stage FG1) or two-nucleate stages (FG2 and FG3). The earliest stage at which abnormal female gametophytes were observed was the four-nucleate stage (FG4). At this stage, a small proportion of embryo sacs contained two normal nuclei and two degenerated nuclei (Fig. 2*F*). Generally, the two degenerated nuclei were those at the chalazal pole (Fig. 2*F*). By the eight-nucleate stage (beginning at stage FG5), half of the female gametophytes within the ovary of a heterozygous plant were abnormal, indicating full penetrance at these stages. Abnormal female gametophytes had pleiotropic defects in cell morphology and nuclear position (Fig. 2 *G* and *H*). With reference to the cell polarity characteristic of a wild-type female gametophyte, nuclei were positioned inappropriately, both within the cells and relative to the other cells. Cell vacuoles were also misshapen and out of place. Taken together, these data indicate that the *CKI1* gene product is essential for normal cell morphology, as well as control over the number of nuclear divisions, during megagametogenesis.

The defect in megagametogenesis of *cki1* mutants strongly indicates that the *CKI1* gene is expressed within the female gametophyte. To test this prediction, we carried out RNA *in situ* hybridization experiments with developing ovules. The *CKI1* probe produced a weak signal that was ≤1/10 that of the control *FILAMENTOUS FLOWER* probe, indicating that *CKI1* RNA is present at low levels in developing ovules. Within mature female gametophytes, the *CKI1* probe consistently produced a strong

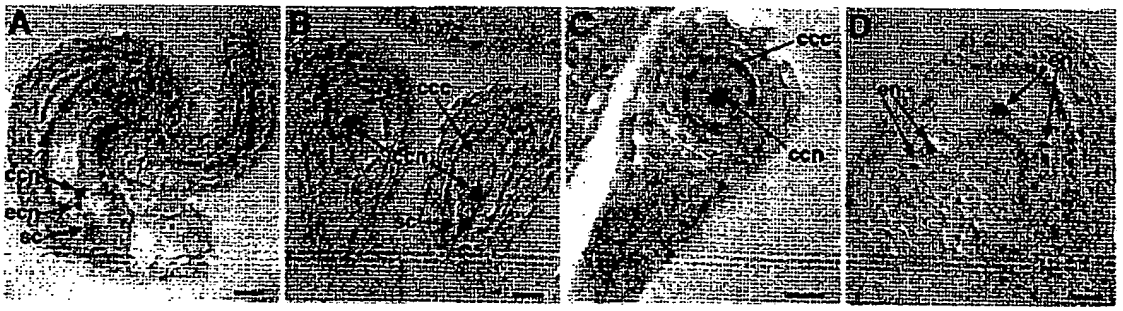

Fig. 3. *CKI1* expression in the female gametophyte and endosperm. (*A–C*) Unfertilized ovules. A strong signal is associated with the nucleus of the central cell, and a weak signal with the nucleus of the egg cell. Note that no signal is associated with the synergid cells in *A* but a weak signal is associated with the synergid cells in *B*. (*D*) Fertilized ovule. A strong signal is associated with some but not all endosperm nuclei. ccc, central cell cytoplasm; ccn, central cell nucleus; ecn, egg cell nucleus; en, endosperm nuclei; sc, synergid cells. (Bar, 10 μm.)

Table 3. Molecular complementation of the *cki1* phenotype

T_0* genotype	Kan^R:Kan^S†	T-DNA transmission‡, %	No. of complemented plants§
cki1-5/CKI1	14:9	60.9	4 (13)
cki1-5/CKI1	17:7	70.8	5 (17)
cki1-5/CKI1	14:9	60.9	ND
cki1-5/CKI1	19:11	63.3	ND
cki1-5/CKI1	14:6	70.0	ND
cki1-5/CKI1	15:6	71.4	ND
cki1-5/CKI1	21:13	61.8	ND
cki1-5/CKI1	18:11	62.1	ND
cki1-5/CKI1	20:11	64.5	ND
cki1-5/CKI1	20:8	71.4	ND
cki1-5/CKI1	19:9	67.9	ND
cki1-5/CKI1	19:8	70.4	ND
cki1-5/CKI1	24:11	68.6	ND
cki1-5/CKI1	15:9	62.5	ND
cki1-5/CKI1	20:11	64.5	ND
cki1-5/CKI1	19:11	63.3	ND
cki1-6/CKI1	16:7	69.6	3 (16)
cki1-6/CKI1	13:6	68.4	ND
cki1-6/CKI1	14:7	66.7	ND
cki1-6/CKI1	16:7	69.6	5 (15)
cki1-6/CKI1	20:9	69.0	ND
cki1-6/CKI1	21:9	70.0	ND

Each row describes an independent transgenic line. ND, not determined.
*T_0 refers to the plant whose progeny was transformed.
†Kanamycin segregation data for the T_2 generation of transformed plants.
‡Percent T-DNA transmission was calculated as $100 \times Kan^R/(Kan^R + Kan^S)$.
§Complemented plants were determined by PCR to have the genotype *cki1/cki1 + CKI1* transgene. Numbers in parentheses indicate the total number of Kan^R plants genotyped.

signal in the central cell nucleus and a weaker signal in the egg cell nucleus (Fig. 3 *A–C*). *CKI1* RNA was not detected in the cytoplasm of these two cells (Fig. 3 *B* and *C*). Occasionally, a weak signal was associated with the synergid cells (seen in Fig. 3*B* but not in *A*). Although the phenotypic abnormalities in a *cki1* female gametophyte are apparent by stage FG5, we did not detect a *CKI1* signal in developing embryo sacs at stages before FG7 (data not shown), indicating lower RNA levels were present during those stages.

We also analyzed *CKI1* expression in fertilized ovules at 24–48 h after pollination. In fertilized ovules, a strong signal was present in the endosperm nuclei (Fig. 3*D*). Not all endosperm nuclei within a given ovule displayed a signal. This observation indicates that *CKI1* expression may be under cell cycle control (30). *CKI1* RNA was not detected in embryos by means of *in situ* hybridization.

We were able to detect *CKI1* RNA by performing RT-PCR on RNA isolated from pistils containing mature female gametophytes or young siliques (data not shown). We were not usbable to detect a *CKI1* transcript by performing RT-PCR on RNA isolated from 10- to 12-day-old seedlings, callus tissue, or shoot apical meristems. Additionally, we saw a very weak signal within the entire embryo sac of plants expressing a *CKI1* promoter::GFP reporter fusion (data not shown).

The mRNA expression data, combined with the availability of two independent insertion alleles of *cki1* with identical phenotypes, provides strong evidence that the defect in a *cki1* female gametophyte is caused by a disruption in the *CKI1* gene. To further establish a causal link between genotype and phenotype, we used molecular complementation to identify plants homozygous for an insertion in *CKI1*, yet viable because of the presence of a *CKI1* transgene. A wild-type copy of *CKI1* was introduced, along with a gene conferring BASTA resistance, into Kan^R *cki1-5/CKI1* and *cki1-6/CKI1* plants (generation T_0) by means of T-DNA-mediated transformation. BASTA-resistant plants were recovered, and their progeny (generation T_2) was screened for evidence of complementation. First, we analyzed the segregation of kanamycin resistance in the T_2 generation. As described, the progeny resulting from self-fertilization of a *cki1/CKI1* plant showed a 1:1 ratio of Kan^R:Kan^S. If the female gametophyte lethality is successfully complemented by the introduced *CKI1* gene, the *cki1* mutant allele should be transmitted through the female gametophyte as well as the male gametophyte. Therefore, we would expect to recover Kan^R progeny at a higher frequency in these lines. In most cases, the *CKI1* transgene will segregate independently of the *CKI1* locus, and if one copy of the transgene is present, we would expect to rescue the *cki1* defect 50% of the time. Thus, we were looking for a bias toward Kan^R plants on the order of 2:1 Kan^R:Kan^S in the T_2 generation. As seen in Table 3, several independent transgenic lines originating from both *cki1-5/CKI1* and *cki1-6/CKI1* T_0 plants show a Kan^R:Kan^S ratio consistent with successful complementation of the *cki1* homozygous lethal phenotype. Four lines were selected for further analysis. In each case, PCR genotyping confirmed the existence of individual plants homozygous for either the *cki1-5* or *cki1-6* insertion allele, yet viable because of the presence of the *CKI1* transgene (Table 3).

Discussion

We described the isolation of two independent T-DNA insertion alleles that disrupt the function of a specific histidine kinase gene, *CKI1*. We provided several lines of evidence that, taken together, support a role for CKI1 in megagametogenesis: failure to recover a plant homozygous for the mutation, distortion from a 3:1 Mendelian segregation to a 1:1 segregation, evidence that mutant alleles are transmitted through the male gametophyte but not the female gametophyte, a ratio of 1:1 wild-type to mutant female gametophytes based on a CLSM analysis of megagametophytes within *cki1/CKI1* pistils, localization of *CKI1* mRNA expression in the developing female gametophyte and endosperm, and complementation of the *cki1* homozygous lethality with a wild-type copy of *CKI1*.

The earliest stage at which phenotypic abnormalities could be detected in a *cki1* female gametophyte is stage FG4, the four-nucleate stage. By this stage, meiosis, haploid megaspore degeneration, and two rounds of mitosis have occurred (21), which indicates that CKI1 function is not required for these processes. From the completion of stage FG4 through stage FG5, the stage at which the *cki1* phenotype is completely penetrant, several important developmental steps occur: a final round of mitosis, cellularization, vacuole formation, and the establishment of cell identities (21). Because of the pleiotropic nature of the *cki1* defect, we are unable at this time to limit the importance of CKI1 function to one of these processes. Megagametophyte-specific promoter::reporter gene fusions may be useful in assigning cellular identities to the abnormal cell types seen in FG5/6 female gametophytes; however, megagametophyte-specific promoters are uncharacterized at this time.

The same phenotypic abnormalities, most commonly, the collapse and degeneration of the embryo sac, were seen in both *cki1-5* and *cki1-6* female gametophytes at the terminal developmental stage. Degeneration of the embryo sac has also been described in another female gametophyte-specific mutant that is nonallelic to *cki1*, the ethyl methanesulfonate-generated *fem1* mutant (22). Although the gene affected in the *fem1* mutant is not known, we can rule out the possibility that *FEM1* is allelic to *CKI2*, another histidine kinase-encoding gene that was identified in Kakimoto's (10) T-DNA activation screen for cytokinin independent mutants, based on mapping information (22). Whether or not *CKI1* and/or *CKI2* actually plays a role in

cytokinin signaling, and what role cytokinins may play in female gametophyte development, remains to be seen. Currently, neither cytokinin levels nor the expression of cytokinin-inducible genes in the *Arabidopsis* embryo sac is known.

Given that specific molecules directing or modulating female gametophyte development have not yet been discovered, it is particularly exciting that a protein kinase with a putative extracellular ligand-binding domain and cytosolic signaling domain has now been implicated in this pathway. Elucidation of the ligand that interacts with CKI1 is an important objective for future study. Cytokinin is unlikely to be the ligand for CKI1 for several reasons. The lack of homology between the putative extracellular domain of CKI1 and that of the cytokinin receptor CRE1, and its two close homologues AHK2 and AHK3 (7), argues against a role for CKI1 as a cytokinin receptor. Membranes isolated from fission yeast expressing *CKI1* do not bind radiolabeled cytokinin (8). Furthermore, the expression of *CKI1* in *Escherichia coli* lacking the histidine kinase RcsC (8) and in *Arabidopsis* protoplasts coexpressing a cytokinin-inducible reporter gene (31) has been shown, in both cases, to activate histidine-kinase-signaling pathways in a constitutive, rather than cytokinin-dependent, manner. Thus, it seems plausible that the overexpression of CKI1 in *Arabidopsis* callus tissue created promiscuous "crosstalk," i.e., allowed the protein to interact with other components of the cytokinin-signaling pathway with which it normally would not interact. Further studies are clearly needed to resolve this question.

Yeast two-hybrid studies by Urao *et al.* (13) have shown that CKI1 can interact with the HPt homologues ATHP1 and ATHP2 *in vitro*. In addition, work by Nakamura *et al.* (32) has shown that the response-regulator domain of CKI1 can act as a phosphatase when incubated with purified, radioactively phosphorylated HPts ATHP1 and ATHP3. Thus, the potential for a His/Asp phosphorelay initiated by CKI1 and involving other His/Asp phosphorelay components certainly exists. The T-DNA insertion mutants *cki1-5* and *cki1-6* can provide the framework for elucidating the *in situ* mechanisms of this first known signal-transduction pathway that operates in the female gametophyte.

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In planta functions of the *Arabidopsis* cytokinin receptor family

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Since their discovery as cell-division factors in plant tissue culture about five decades ago, cytokinins have been hypothesized to play a central role in the regulation of cell division and differentiation in plants. To test this hypothesis *in planta*, we isolated *Arabidopsis* plants lacking one, two, or three of the genes encoding a subfamily of histidine kinases (*CRE1*, *AHK2*, and *AHK3*) that function as cytokinin receptors. Seeds were obtained for homozygous plants containing mutations in all seven genotypes, namely single, double, and triple mutants, and the responses of germinated seedlings in various cytokinin assays were compared. Both redundant and specific functions for the three different cytokinin receptors were observed. Plants carrying mutations in all three genes did not show cytokinin responses, including inhibition of root elongation, inhibition of root formation, cell proliferation in and greening of calli, and induction of cytokinin primary-response genes. The triple mutants were small and infertile, with a reduction in meristem size and activity, yet they possessed basic organs: roots, stems, and leaves. These results confirm that cytokinins are a pivotal class of plant growth regulators but provide no evidence that cytokinins are required for the processes of gametogenesis and embryogenesis.

Since the discovery of kinetin in 1956 as a degradation product of DNA that promotes cell division in plants (1), a considerable amount of biochemical, physiological, and, most recently, genetic research has focused on elucidating the diverse roles that cytokinins play in plant growth and development. Perturbations of cytokinin levels in plants via over-expression of bacterial cytokinin synthesis genes (2–4), recovery of mutant plants with a higher-than-normal cytokinin content (5), and characterization of loss-of-function mutants of the cytokinin receptor CYTOKININ RESPONSE 1 (CRE1) (6–9) have implicated cytokinins in a wide variety of processes, including cell division, organ formation and regeneration, senescence, apical dominance, vascular development, response to pathogens, and nutrient mobility. These numerous roles for cytokinins, coupled with the failure of mutant screens to yield plants with nondetectable cytokinin levels, led to the longstanding belief that cytokinins are essential for plant growth and development.

Plants respond to cytokinin through a multistep phosphorelay system, consisting of sensor histidine kinase (HK) proteins, histidine phosphotransfer (HPt) proteins, and effector response regulator (RR) proteins. Over-expression and loss-of-function analyses of particular HK, HPt, and RR proteins in *Arabidopsis* (8–13), combined with transient expression assays in protoplasts (14), have led to a model for cytokinin signaling (for a review, see refs. 15 and 16), beginning with perception of cytokinins by HK proteins.

The *Arabidopsis* genome encodes six nonethylene receptor HKs: CRE1/WOL/AHK4, AHK2, AHK3, AtHK1, CKI1, and CKI2/AHK5. Among them, CRE1, *Arabidopsis* HK2 (AHK2), and *Arabidopsis* HK3 (AHK3) (hereafter called the CRE family) are highly homologous at the amino acid level, especially within the putative cytokinin-binding extracellular domain (≈60% identity). CRE1 was the first cytokinin-signaling component identified. A substantial body of evidence supports a role for CRE1 as a cytokinin receptor: plants carrying loss-of-function mutations in the *CRE1* gene have a reduced sensitivity to cytokinin (8, 9), CRE1 initiates a phosphorelay in response to cytokinins when expressed in heterologous systems (8, 9, 17), and fission yeast expressing CRE1 bind active cytokinins in a specific and saturable manner (18). Similar to CRE1, AHK2 (M.H. and T.K., unpublished data) and AHK3 (18) are also activated by cytokinins when expressed in yeast and bacteria, respectively.

CKI1 was first identified as a gene that induces constitutive cytokinin responses when overexpressed in callus tissue (19). However, all subsequent efforts to detect cytokinin receptor activity of CKI1 have yielded negative results (refs. 14 and 18 and T.K., unpublished work). CKI2 is the only HK lacking a putative extracellular domain, and loss-of-function mutants of *CKI2* have no noticeable phenotype (Y.H. and T.K., unpublished work). AtHK1 has been implicated in osmosensing (20), and the HK activity of AtHK1 in yeast is unchanged by cytokinins. These results indicate that CRE-family members are cytokinin receptors, but that the other nonethylene receptor HKs are less likely to perform a cytokinin-sensing role.

To elucidate the *in planta* roles of the three CRE-family members, as well as the role of cytokinin signaling in plant development, we identified loss-of-function alleles for *CRE1*, *AHK2*, and *AHK3*; created plants containing all possible mutant allele combinations; and characterized their responses in a series of cytokinin assays. These observations are discussed in relation to the relative contributions of each family member to cytokinin action, as well as the overall role of cytokinins in plant growth and development.

Materials and Methods

Plant Materials and Growth Conditions. Unless otherwise indicated, sterilized seeds of *Arabidopsis thaliana*, ecotype Columbia (Col), were incubated at 4°C for 2 days on germination medium (GM) containing full-strength Murashige and Skoog salts (MS) (21); 0.05% (wt/vol) Mes-KOH (pH 5.7); 1% (wt/vol) sucrose; 100 mg/ml inositol; 10 mg/ml thiamine HCl; 1 mg/ml nicotinic acid; 1 mg/ml pyridoxine HCl; and 0.3% (wt/vol) Phytagel (Sigma–

Abbreviations: HK, histidine kinase; AHK2, *Arabidopsis* HK2; AHK3, *Arabidopsis* HK3; BA, benzyl adenine; Col, Columbia; CRE1, cytokinin response 1; SAM, shoot apical meristem; GM, germination medium; MS, Murashige and Skoog salts; GUS, β-glucuronidase; *t*-zeatin, *trans*-zeatin.

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Aldrich); and grown at 22°C under constant illumination (100 μmol/m²·s). For plastochron measurements, the number of leaves >0.5 mm in width from 11-day-old seedlings was counted under a dissecting microscope. For adventitious root formation assays as well as callus and shoot induction assays, 11-day-old plants grown aseptically under dim light (2.5 μmol/m²·s) were used. For visualization of root nuclei, roots from 21-day-old plants were fixed with 3.7% (vol/vol) formaldehyde, stained with 1 μg/ml 4',6-diamidino-2-phenylindole (DAPI), and photographed on an Olympus BX50 (Melville, NY) microscope with a Roper Scientific Coolsnap HQ/OL digital camera. For analysis of shoot apical meristem (SAM) morphology, seeds were germinated on full-strength MS, 4.5% (wt/vol) sucrose, 0.05% (wt/vol) Mes, and 0.8% (wt/vol) agar (MS + 4.5 suc), and grown for 7 days. Seedlings were fixed, embedded, and sectioned according to Mähönen *et al.* (6). Three-micrometer sections were stained with 0.05% (wt/vol) toluidine blue in water and photographed on an Olympus Provis microscope with an Olympus DP70 digital camera.

Cytokinin Response Assays. For the root elongation assay, seedlings grown under constant light for 8 days on GM supplemented with 1/5,000 volume of appropriate concentrations of benzyl adenine (BA) dissolved in DMSO were removed from plates, and root lengths were measured. Plants that had not germinated within 2 days of culture were excluded from the analysis. For the adventitious root formation assay, plants were separated into upper and lower portions by bisecting the hypocotyl with fine scissors. The upper portions were inserted into GM supplemented with 1/5,000 volume of appropriate concentrations of *trans* (*t*)-zeatin dissolved in DMSO. After 11 days, the presence or absence of adventitious roots near the cut site was observed under a dissecting microscope. For the callus induction assay, hypocotyls were excised with fine scissors and cultured for 24 days on GM supplemented with 30 ng/ml 2,4-dichloriphenoxyaceticacid (2,4-D) and varying concentrations of kinetin. All plates contained 0.02% (vol/vol) DMSO. For the shoot formation assay, excised hypocotyls were cultured for 7 days on GM supplemented with 500 ng/ml 2,4-D and 50 ng/ml kinetin to induce callus formation. Calli were then moved onto GM supplemented with 0.3 μg/ml indolebutyric acid and varying concentrations of *t*-zeatin and cultured for 14 days.

Expression Patterns of Cytokinin Receptor Genes. For RNA gel blot analysis, 5 μg of total RNA was used per lane. DNA fragments corresponding to a region of *CRE1*, *AHK2*, or *AHK3* were amplified by PCR. Primer sequences are available in *Supporting Materials and Methods*, which is published as supporting information on the PNAS web site. After the T7 promoter was ligated to the 3' end of each amplified fragment, radiolabeled riboprobes were synthesized by using the MAXIscript T7 Kit (Ambion, Austin, TX). Hybridizations were conducted in PerfectHyb (Toyobo, Dojima, Osaka) according to the manufacturer's instructions. For expression patterns of reporter genes, the promoter sequence for each of the CRE-family genes was cloned in-frame upstream of the β-*glucuronidase* (*GUS*) gene. Details of the reporter gene cloning are available in the *Supporting Materials and Methods*. *CRE1*::*GUS* and *AHK3*::*GUS* were introduced into *Arabidopsis*, ecotype Wassilewskija (Ws), by the floral dip method (22). *AHK2*::*GUS* was similarly introduced into *Arabidopsis* (Col). Expression patterns for each gene were determined according to Miyawaki *et al.* (23).

Screening for T-DNA Insertion Mutants. Multiple alleles of T-DNA insertion mutants were identified. One set of mutants (*cre1–10*, *ahk2–1*, *ahk3–1*) is in the ecotype Ws, whereas another set of mutants (*cre1–12*, *ahk2–2*, and *ahk3–3*) is in the ecotype Col. Double and triple mutants were generated in the same backgrounds. Additional mutant alleles identified in the ecotype Ws include *ahk3–2* and *cre1–11*. Details of the screening process are provided in *Supporting Materials and Methods*.

Expression of Cytokinin Receptor Genes in Mutant Backgrounds. Details of the RT-PCR analysis of CRE-family genes in *cre1–12*, *ahk2–2*, and *ahk3*-3 mutant plants are available in *Supporting Materials and Methods*.

Expression of Cytokinin Primary-Response Genes. For real-time quantitative PCR (qRT-PCR) analysis of cytokinin-inducible gene expression, seeds were germinated on MS + 4.5suc and grown for 6 days. Cytokinin treatment was carried out by incubating seedlings in an MS + 4.5suc solution without agar and supplemented with 10 μM BA for 30 min. Before RNA preparation, three WT Col and five *cre1–12 ahk2–2 ahk3–3* (Col) seedlings were pooled and stored in RNAlater solution (Qiagen, Valencia, CA). Total RNA was extracted by using the RNeasy Plant Mini Kit (Qiagen). TaqMan RT-PCR reagents (Applied Biosystems) were used to synthesize double-stranded cDNA. Unlabeled gene-specific primers and 6-carboxy-fluorosceinlabeled gene-specific TaqMan Minor Groove Binder probes were used for qRT-PCR with ABI prism 7700 (Applied Biosystems). The number of *ARR5* and *ARR15* (*Arabidopsis Response Regulator*) transcripts present in two biological replicates each of WT, and *cre1–12 ahk2–2 ahk3–3* seedlings, with or without BA, was determined three separate times. Fold induction of the *ARR5* and *ARR15* cytokinin primary-response gene transcripts was calculated relative to the *SHORT ROOT* (*SHR*) transcript (24), according to the manufacturer's instructions (ABI Prism 7700 Sequence Detection System, User Bulletin #2). Primer and probe sequences are available in *Supporting Materials and Methods*.

Flow Cytometry. Experiments were performed according to Shpak *et al.* (25), with some modifications. Plants were grown vertically on GM plates, with 1.8% (wt/vol) purified agar. Whole roots of 33-day-old plants were finely chopped in 0.5 ml of ice-cold extraction buffer [15 mM Hepes/1 mM EDTA/80 mM KCl/20 mM NaCl/300 mM sucrose/0.5% (vol/vol) Triton X-100/0.5 mM spermine/0.1% (vol/vol) 2-mercaptoethanol), passed through 33-μm nylon mesh, and centrifuged at 3,000 × *g* for 1 min. The pellet was resuspended in 100 μl of staining buffer (1/10,000 dilution of SYBR green I (Molecular Probes)/50 μg/ml RNase A/3.7% (vol/vol) formaldehyde in the extraction buffer) and subjected to FACScan (Becton Dickinson) by using the FL2 channel with a photomultiplier voltage of 300 V.

Results

Expression Patterns of Cytokinin Receptor Genes. RNA gel blot hybridization experiments confirmed that the three CRE-family genes have distinct expression patterns (Fig. 1*A*). *CRE1* expression was highest in the root and low in rosette leaves. *AHK2* was expressed to about the same degree in rosette leaves and the root. *AHK3* expression was highest in rosette leaves, moderate in the root, and low in the silique. Expression of all three cytokinin receptor genes was detected in flowers. Differential expression of the cytokinin receptor genes in root and shoot tissue was confirmed by expression of the *GUS* reporter gene directed by regulatory sequences from *CRE1*, *AHK2*, or *AHK3*. CRE1::GUS activity was high in the root, moderate in the inflorescence stems and pedicels, and low in the leaves (Fig. 1 *B* and *E*). AHK2::GUS activity was high in leaf veins, petioles, inflorescence stems, flowers, and siliques, and moderate in the roots (Fig. 1 *C* and *F*). AHK3::GUS was expressed ubiquitously in root and shoot tissues including leaves, inflorescence stems, and flowers (Fig. 1 *D* and *G*).

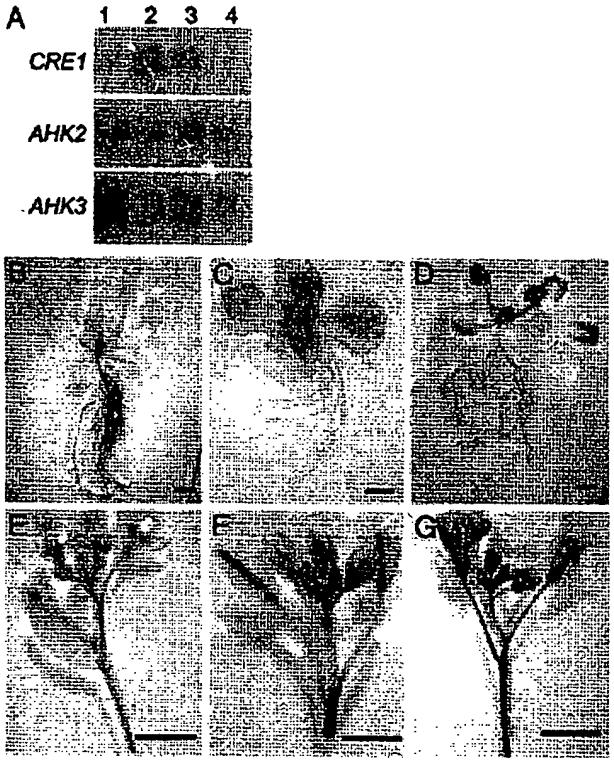

Fig. 1. Expression patterns of the *CRE*-family genes. (*A*) RNA gel blot hybridizations (5 μg of RNA) from different tissues of WT plants, probed with gene-specific probes for *CRE1*, *AHK2*, or *AHK3*. 1, rosette leaves; 2, roots; 3, floral bunches; 4, siliques. (*B*) Expression of the GUS reporter gene under control of regulatory sequences from cytokinin-receptor genes. (*B* and *E*) CRE1::GUS activity. (*C* and *F*) AHK2::GUS activity. (*D* and *G*) AHK3::GUS activity. (Bars = 2 mm.)

Expression of Cytokinin Receptor Genes in the Mutant Background. Multiple independent T-DNA insertion alleles for all members of the *Arabidopsis* CRE cytokinin receptor family (*AHK2*, *AHK3*, and *CRE1*) were identified (Fig. 2). To determine whether full-length transcripts of *CRE1*, *AHK2*, and *AHK3* were present in the T-DNA insertion mutants, RT-PCR analysis was performed on RNA prepared from plants carrying a single mutation in the CRE-family genes by using gene-specific primers flanking the T-DNA insertion site (see Fig. 10, which is published as supporting information on the PNAS web site). Even

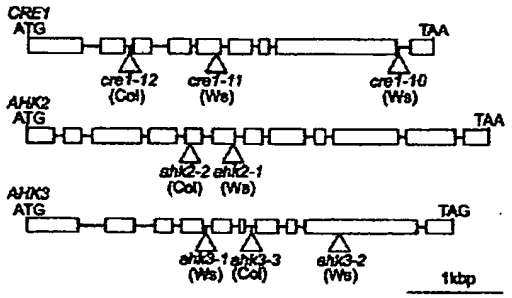

Fig. 2. Description of the *CRE1*, *AHK2*, and *AHK3* T-DNA insertion alleles. Boxes represent exons; horizontal bars, introns; and triangles, T-DNA integration sites.

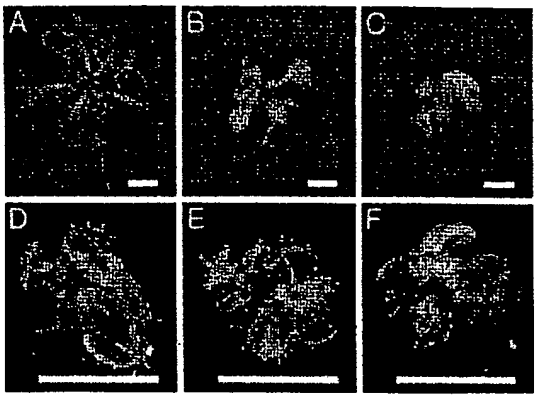

Fig. 3. Phenotype of the triple mutant grown with or without *t*-zeatin. (*A–C*) Thirteen-day-old WT seedlings from the ecotype Col. (*D–F*) Thirteen-day-old *cre1-12 ahk3-3 ahk2-2* (Col) triple mutants. Plants were grown on plates with 0 ng/ml (*A* and *D*), 100 ng/ml (*B* and *E*), 1,000 ng/ml *t*-zeatin (*C* and *F*). (Bars = 5 mm.)

with saturating numbers of PCR cycles, the full-length transcripts of *CRE1* and *AHK2* were not detected in *cre1-12* and *ahk2-2* mutants, respectively (Fig. 10*A*). The full-length transcript of *AHK3* was not detected in the *ahk3-3* mutant with the same number of PCR cycles that yielded near-saturating amplification of *AHK3* in WT plants (Fig. 10*A*). However, the transcript was detected with a greater number of PCR cycles (data not shown). The full-length transcripts of *CRE1*, *AHK2*, or *AHK3* were not detected in *cre1-10* or *cre1-11*, *ahk2-1*, *ahk3-1*, or *ahk3-2* mutants, respectively (data not shown). Recovery of *Arabidopsis* plants containing multiple independent T-DNA insertion alleles for each of the CRE-family genes, lacking expression of a full-length transcript, indicates that these T-DNA insertion mutants are null alleles. The greatly reduced level of *AHK3* expression detected in the *ahk3-3* mutant is unlikely to contribute to cytokinin signaling, because the cytokinin-response phenotypes of the *ahk3-3* mutant are the same as those observed for the *ahk3-1* and *ahk3-2* null mutants. Likewise, the cytokinin-response phenotypes of the *cre1-12 ahk3-3* and *ahk2-2 ahk3-3* double mutants are the same as those observed for the *cre1-10 ahk3-1* and *ahk2-1 ahk3-1* double mutants, respectively.

Overall Appearance of Cytokinin Receptor Mutants. When grown on soil, plants with a single mutation in *AHK2*, *AHK3*, or *CRE1* grew normally (see Fig. 11, which is published as supporting information on the PNAS web site). When grown on soil, plants carrying the *cre1-12 ahk2-2*, *cre1-12 ahk3-3* (Fig. 11), *cre1-10 ahk2-1*, and *cre1-10 ahk3-1* (not shown) mutations exhibited no noticeable phenotype. The *ahk2-2 ahk3-3* (Fig. 11) and *ahk2-1 ahk3-1* (not shown) double mutants had smaller leaves and shorter stems than did the WT plants. This result indicates that *AHK2* and *AHK3* functions dominate in the shoot. Roots of double mutants for any mutant combination were normal. Surprisingly, triple mutants were recovered in both the Ws background (*cre1-10 ahk2-1 ahk3-1*, and *cre1-11 ahk2-1 ahk3-1*) (not shown) and the Col background (*cre1-12 ahk2-2 ahk3-3*) (Figs. 3*D* and 11), indicating that seeds can germinate and seedlings can grow for a limited period without any of the *CRE-family* genes being expressed. The shoot and root growth of triple mutants was very slow, and leaf numbers were decreased (see below). The triple mutants occasionally produced an inflorescence stem with abnormal and nonfunctional flowers, but did not produce seeds. Supplementing media with 1 μg/ml *t*-zeatin

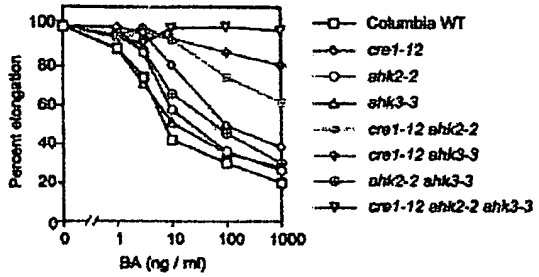

Fig. 4. Elongation of roots of cytokinin-receptor mutants in the presence of increasing concentrations of BA. Root length of each genotype without cytokinin was set at 100%. Lengths of roots in the absence of BA were: WT, 2.58 ± 0.29 (mean ± SD); *cre1–12*, 2.65 ± 0.32; *ahk2–2*, 2.18 ± 0.28; *ahk3–3*, 2.81 ± 0.38; *cre1–12 ahk2–2*, 2.57 ± 0.38; *cre1–12 ahk3–3*, 3.01 ± 0.35; *ahk2–2 ahk3–3*, 2.69 ± 0.35; and cre1–12 *ahk2–2 ahk3–3*, 0.65 ± 0.09.

severely inhibited the growth of WT seedlings but did not affect the growth of triple mutants (Fig. 3), suggesting that the triple mutants lack a mechanism for cytokinin perception. The insensitivity of the triple mutants to cytokinins was verified with several cytokinin-response assays, as shown below.

Cytokinin Sensitivity in the Root Growth Assay. Exogenous cytokinins normally inhibit root elongation. As previously reported (8), mutations in *CRE1* caused a reduced sensitivity to cytokinin (*cre1–12*, Fig. 4; *cre1–10* and *cre1–11*, not shown). The *ahk2–2* or *ahk3–3* mutants exhibited normal or slightly reduced sensitivity (Fig. 4). Additive effects were seen in the double mutants; that is, *cre1–12 ahk2–2*, *cre1–12 ahk3–3*, and *ahk2–2 ahk3–3* double mutants were less sensitive to cytokinin than was either single mutant (Fig. 4). Similarly, *cre1–10 ahk2–1* and *cre1–10 ahk3–1* double mutants were less sensitive than was either single mutant (not shown). The triple mutant had a shorter root with respect to WT plants, and the root length was not affected by BA up to 1 μg/ml. These data indicate that CRE1, AHK2, and AHK3 have redundant functions in cytokinin signaling in roots.

Cytokinin Sensitivity in the Adventitious Root Formation Assay. Cytokinins normally inhibit adventitious root formation near the cut end of hypocotyls (26). The *cre1–12* and *ahk3–3* single mutants were less sensitive to cytokinins in the adventitious root formation assay, whereas the *ahk2–2* mutant exhibited a normal sensitivity (Fig. 5). The effect of mutations in both *CRE1* and *AHK3* was synergistic; that is, *cre1–12 ahk3–3* roots were completely resistant to all cytokinin concentrations tested (up to a nonphysiological concentration of 3 μg/ml *t*-zeatin) (Fig. 5). These data indicate that CRE1 and AHK3 are key regulators of

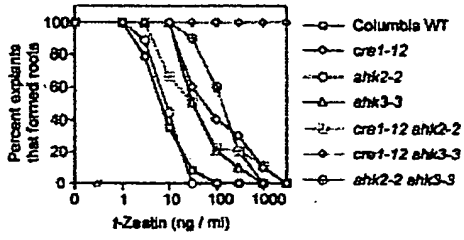

Fig. 5. Cytokinin inhibition of adventitious root formation in cytokinin-receptor mutants. The percent of explants per genotype producing adventitious roots on increasing concentrations of t-zeatin is presented. At least eight plants were used for each data point.

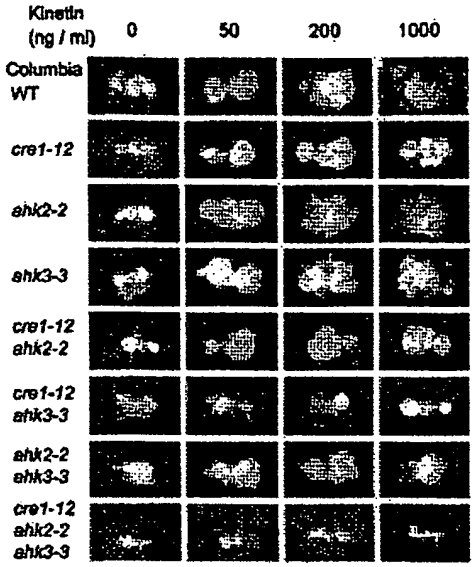

Fig. 6. Induction of callus formation on hypocotyl segments, from cytokinin-receptor mutants, on different concentrations of kinetin.

cytokinin-induced inhibition of adventitious root formation in *Arabidopsis*.

Cytokinin Sensitivity in the Callus Induction Assay. Cytokinins normally stimulate cell division and greening of calli (1). Similar to previously published work (8), cytokinin-induced cell division and greening of hypocotyl-derived calli were partially inhibited in *cre1–12* mutants (Fig. 6), as well as in *cre1–10* and *cre1–11* mutants (data not shown). The *ahk2–2* and *ahk3–3* mutants responded normally to cytokinin in this assay (Fig. 6). Similar results were seen with *ahk2–1*, *ahk3–1*, and *ahk3–2* mutants (data not shown). Mutations in *AHK2* and *AHK3*, in combination with a mutation in *CRE1*, enhanced the effect of the *cre1–12* mutation (Fig. 6). Finally, the triple cytokinin receptor mutant showed no significant response in this assay (Fig. 6). These results indicate that CRE1, AHK2, and AHK3 have redundant function in callus induction.

Cytokinin Sensitivity in the Shoot Formation Assay. Cytokinins induce shoot formation and inhibit root formation on calli (27). The *cre1–12*, *ahk2–2*, and *ahk3–3* single mutants exhibited normal or slightly reduced sensitivity to cytokinins in a shoot induction assay (see Fig. 12, which is published as supporting information on the PNAS web site). Similar results were seen for the *cre1–10*, *cre1–11*, *ahk2–1*, *ahk3–1*, and *ahk3–2* single mutants (data not shown). Additive effects were observed for all CRE-family mutant combinations (Fig. 12). This result indicates that CRE1, AHK2, and AHK3 have redundant functions in cytokinin-induced shoot formation.

Cytokinin Induction of Primary-Response Genes Is Absent in the Triple Mutant. Cytokinins normally induce the transcription of type A response regulator genes in *Arabidopsis* (28, 29). To determine whether induction of these cytokinin primary-response genes was compromised in the triple mutant, reverse transcription, and real-time quantitative PCR analysis was performed on RNA prepared from WT and *cre1–12 ahk2–2 ahk3–3* triple mutants, before and after a 30-min cytokinin treatment. Cytokinin treatment of WT seedlings induced transcription of the *ARR5* and *ARR15* transcripts by ≈14- and 13-fold, respectively (Fig. 7).

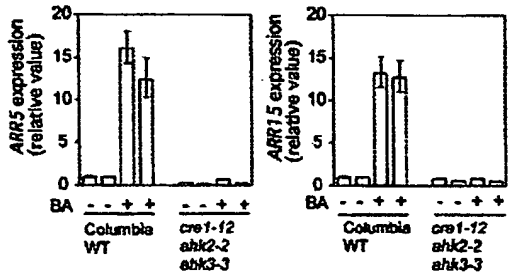

Fig. 7. Induction of cytokinin primary-response genes in the triple mutant. Fold induction of the *ARR5* and *ARR15* (*Arabidopsis Response Regulator*) transcripts in WT (Col) and the *cre1–12 ahk3–3 ahk2–2* (Col) triple mutant, in response to BA, is presented. Results are based on real-time quantitative PCR analysis, and normalized to the *SHR* (*SHORT ROOT*) transcript level.

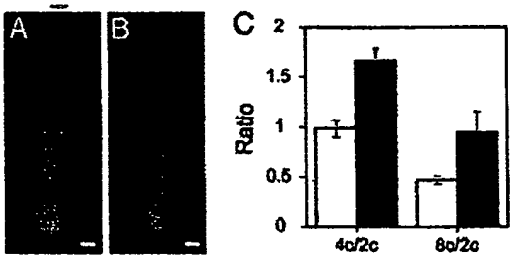

Fig. 9. Root meristem activity of the triple mutant. (*A* and *B*) One microgram per milliliter 4′,6-diamidino-2-phenylindole staining of root cells of WT (Col) (*A*) and *cre1–12 ahk2–2 ahk3–3* (Col) triple mutant (*B*). (Bars = 50 μm.) (*C*) tetraploid (4C/2C) and octaploid (8C/2C) ratios ± standard deviation ($n = 4$). Open column, WT; filled column, *cre1–12 ahk2–2 ahk3–3* mutant.

Cytokinin treatment of the triple mutant produced no change in *ARR5* or *ARR15* transcript levels (Fig. 7).

The Size and Activity of SAMs Are Decreased in the Triple Mutant. The diameter of the SAM was almost three times smaller in the triple mutant (29 ± 7 μm; $n = 5$) when compared to WT (82 ± 7 μm; $n = 9$) (Fig. 8). Fewer cell layers were seen in the SAM of triple mutants, as well as fewer cells per layer. One function of the SAM is to produce leaf primordia. Typically, plants with an enlarged SAM have a shorter interval of leaf production, or more rapid plastochron, than plants with a smaller SAM (5, 30, 31). As expected from the reduced SAM, the triple mutants had a prolonged plastochron with respect to WT plants. The leaf number of the *cre1–12 ahk2–2 ahk3–3* (Col) triple mutants was 4.0 ± 0.0 (mean ± standard deviation, $n = 7$) after 11 days of culture, whereas that of WT (Col) was 9.13 ± 0.83 ($n = 7$). The leaf numbers of single and double mutants were not significantly different from those of WT.

The size and activity of the root apical meristem of the triple mutant were also decreased with respect to WT plants (Fig. 9). The reduction in activity was seen in fluorescence-activated cell sorting experiments of root cells whose DNA had incorporated SYBR green I dye (see Fig. 13, which is published as supporting information on the PNAS web site). As shown in Fig. 9*C*, the triple mutant had a reduced diploid (2C) content of DNA with respect to WT plants, indicating that root cells of the triple mutant are delayed in the transition from $G_2 \rightarrow M$ phase of the cell cycle.

Discussion

The main purpose of this study was to determine whether CRE-family members are the only cytokinin receptors in *Arabidopsis* by identifying a plant lacking expression of all three genes, and noting any developmental consequences, should this plant be viable. Indeed, plants homozygous for a T-DNA insertion in *CRE1*, *AHK2*, and *AHK3* were recovered. The severe developmental abnormalities of these plants confirm that cytokinins are key regulators of plant growth and development. However, the phenotype of the triple mutant calls into question the longstanding belief that cytokinins are essential plant hormones, because an embryo is formed and a seedling produced without expression of the three known cytokinin receptors. It should be noted that our results do not eliminate the possibility that additional yet-unknown cytokinin receptors function in gametogenesis and embryogenesis.

Functions of CRE-Family Genes in the Root. The resistance of *cre1* mutants to cytokinin-induced inhibition of root growth and adventitious root formation indicates that CRE1 functions dominate in the root. Mutations in either *AHK2* or *AHK3* had only minor effects on cytokinin-induced inhibition of root growth. However, either of these mutations in combination with the *cre1* mutation enhanced the effect of *cre1* mutation, suggesting that AHK2, AHK3, and CRE1 have redundant functions in the root. The important roles cytokinins play in root development can best be seen in the triple mutant, which has shorter, narrower roots than do WT plants (data not shown).

The size and activity of the root apical meristem were markedly reduced in the triple mutant. Cytokinins were first discovered for their role in promoting cell division (1) and have since been implicated in stimulating both the $G_1 \rightarrow S$ phase transition (32) as well as the $G_2 \rightarrow M$ phase transition of the cell cycle (33). The reduced meristem activity seen in the triple mutant roots seems to be a consequence of a delay in the $G_2 \rightarrow M$ phase, as demonstrated by FACS experiments. Interestingly, a sharp increase in the levels of zeatin, zeatin riboside, and zeatin riboside-5′-monophosphate was reported in tobacco cell cultures at the $G_2 \rightarrow M$ phase transition (34).

Our data are consistent with a role for cytokinins as positive regulators of root apical meristem activity. This is inconsistent with the findings of Werner *et al.* (35, 36), who demonstrated that over-expression of several members of the *Arabidopsis* cytokinin oxidase family led to an increase in root meristem size and activity. A possible explanation for this difference is to assume that cytokinins have two opposing effects on root growth, one inhibitory and one stimulatory. An inhibitory effect on root elongation could be mediated by cytokinin-induced ethylene production, because it is known that cytokinins induce ethylene production, ethylene inhibits root growth, and roots of ethylene-resistant mutants are also resistant to cytokinins (37). Another role for cytokinins in roots could be as a stimulator of cell division. Most likely, cell-division defects in the root occur only when cytokinin signaling is severely inhibited, as seen in the triple mutant.

Fig. 8. Microscopic analysis of median longitudinal sections from the SAM of WT (Col) (*A*) and the *cre1–12 ahk2–2 ahk3–3* (Col) mutant (*B*). (Bar = 25 μm.)

***CRE1, AHK2, and AHK3* Functions in the Shoot.** The observation that *ahk2 ahk3* double mutants have shorter inflorescence stems and smaller leaves than do WT plants, yet normal root growth, indicates that AHK2 and AHK3 functions dominate in the shoot. The severely stunted growth of the aerial portions of triple cytokinin receptor mutants indicates a redundant function for CRE1 in the shoot as well. Histological analyses showed that the SAM of the triple mutant was 3-fold smaller than normal. The organization of the SAM also appeared to be disrupted, because only the outermost layer of the tunica (L1) was recognizable. The interval of leaf formation was prolonged in the triple mutant, providing evidence of reduced SAM activity. Thus, our results support the finding of Werner *et al.* (35, 36) that cytokinins play a positive role in the SAM.

CRE1, AHK2, and AHK3 Function in *de Novo* Organ Formation. Since the late 1950s, scientists have recognized the ability of relative concentrations of the hormones cytokinin and auxin to induce plant cells to form particular tissues: undifferentiated callus, shoot structures, root structures, or a whole plant (21, 27). The ability to respond appropriately to cytokinin in an organ induction assay was retained in the *cre1*, *ahk2*, and *ahk3* single mutants, suggesting redundant functions for the CRE-family members in organ formation. Among the single mutants, callus formation was most compromised in the *cre1* mutant, indicating that CRE1 may play an important role in the process of dedifferentiation. The ability to appropriately respond to cytokinin in callus and shoot induction assays was lost in all three double mutant combinations, indicating that no single cytokinin receptor is sufficient for organ formation. Strikingly, the triple mutant did not respond to cytokinins at all, indicating that there may be no other cytokinin receptors that function in cell division and differentiation under tissue culture conditions.

What Is the Role of Cytokinins in the Formation of a Basic Vegetative Body Plan? The complete lack of cytokinin responses in the triple mutant, including the absence of cytokinin primary-response gene induction, could suggest that no other mechanism for cytokinin sensing exists in these plants. The retarded growth and sterility of the triple mutants indicate that cytokinins are very important growth regulators. In light of the inability of the triple mutant to form organs in tissue culture, that these plants can germinate and produce the basic plant organs induced *in vitro* by cytokinin and auxin is surprising. One possible explanation for this result is that, despite the well documented requirement for cytokinin in organogenesis during tissue culture, cytokinin-mediated regulation may not be required *in planta* for the formation of a basic vegetative body plan. Less radical interpretations include that there is another cytokinin receptor important for the earliest stages of plant development, or that the cytokinin production and responses of maternal tissues are sufficient for gametogenesis, embryogenesis, and germination of diploid offspring.

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the application of	:
KAKIMOTO, Tatsuo, *et al.*	: GROUP ART UNIT:
Application No.: 09/918508	: Examiner:
Filed: August 1, 2001	:

DECLARATION UNDER 37 C.F.R. § 1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

We, Tomohiko KATO and Satoshi TABATA, state that:

1. We are not joint co-inventors or co-applicants of the subject matter for the invention entitled "ANALYSIS OF AGONIST-ACTIVITY AND ANTAGONIST-ACTIVITY TO CYTOKININ RECEPTOR" as disclosed and claimed in the above-identified patent application, U.S. Application No. 09/918508, filed on August 1, 2001.

2. We are listed as co-authors of a letter to nature present in NATURE, VOL 409, entitled "Identification of CRE1 as a cytokinin receptor from *Arabidopsis*" filed on October 16, 2000 and published on February 22, 2001.

3. We are listed as co-authors of said letter to nature present in NATURE, VOL 409, as a general matter of courtesy and are not co-inventors of the subject matter disclosed in said letter to nature or disclosed and claimed in the aforementioned patent application.

We hereby declare that all of the statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like are so made are punishable by fine or imprisonment, or both,

under § 1001 of Title 18 of the United States Code and that such willful and false statements may jeopardize the validity of the aforementioned patent application and of any patent issued thereon.

Date: August 28, 2001 Signed: Tomohiko Kato
Tomohiko KATO

Date: August 28, 2001 Signed: Satoshi Tabata
Satoshi TABATA

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the application of	:
KAKIMOTO, Tatsuo, *et al.*	: GROUP ART UNIT:
Application No.: 09/918508	: Examiner:
Filed: August 1, 2001	:

DECLARATION UNDER 37 C.F.R. § 1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

We, Motoaki SEKI, Masatomo KOBAYASHI and Kazuo SHINOZAKI state that:

1. We are not joint co-inventors or co-applicants of the subject matter for the invention entitled "ANALYSIS OF AGONIST-ACTIVITY AND ANTAGONIST-ACTIVITY TO CYTOKININ RECEPTOR" as disclosed and claimed in the above-identified patent application, U.S. Application No. 09/918508, filed on August 1, 2001.

2. We are listed as co-authors of a letter to nature present in NATURE, VOL 409, entitled "Identification of CRE1 as a cytokinin receptor from *Arabidopsis*" filed on October 16, 2000 and published on February 22, 2001.

3. We are listed as co-authors of said letter to nature present in NATURE, VOL 409, as a general matter of courtesy and are not co-inventors of the subject matter disclosed in said letter to nature or disclosed and claimed in the aforementioned application.

We hereby declare that all of the statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false

statements and the like are so made are punishable by fine or imprisonment, or both, under § 1001 of Title 18 of the United States Code and that such willful and false statements may jeopardize the validity of the aforementioned patent application and of any patent issued thereon.

Date: August 28, 2001 Signed: Motoaki Seki
Motoaki SEKI

Date: August 28, 2001 Signed: Masatomo Kobayashi
Masatomo KOBAYASHI

Date: August 28, 2001 Signed: Kazuo Shi
Kazuo SHINOZAKI

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the application of	:
KAKIMOTO, Tatsuo, *et al.*	: GROUP ART UNIT:
Application No.: 09/918508	: Examiner:
Filed: August 1, 2001	:

DECLARATION UNDER 37 C.F.R. § 1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, Yukari HASHIMOTO, state that:

1. I am not a joint co-inventor or co-applicant of the subject matter for the invention entitled "ANALYSIS OF AGONIST-ACTIVITY AND ANTAGONIST-ACTIVITY TO CYTOKININ RECEPTOR" as disclosed and claimed in the above-identified patent application, U.S. Application No. 09/918508, filed on August 1, 2001.

2. I am listed as a co-author of a letter to nature present in NATURE, VOL 409, entitled "Identification of CRE1 as a cytokinin receptor from *Arabidopsis*" filed on October 16, 2000 and published on February 22, 2001.

3. I am listed as a co-author of said letter to nature present in NATURE, VOL 409, as a general matter of courtesy and am not a co-inventor of the subject matter disclosed in said letter to nature or disclosed and claimed in the aforementioned patent application.

I hereby declare that all of the statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like are so made are punishable by fine or imprisonment, or both, under § 1001

of Title 18 of the United States Code and that such willful and false statements may jeopardize the validity of the aforementioned patent application and of any patent issued thereon.

Date: September 10, 2001 Signed: Yukari Hashimoto

Yukari HASHIMOTO

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the application of	:
KAKIMOTO, Tatsuo, *et al.*	: GROUP ART UNIT:
Application No.: 09/918508	: Examiner:
Filed: August 1, 2001	:

DECLARATION UNDER 37 C.F.R. § 1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

We, Tatsuo KAKIMOTO, Tsutomu INOUE and Masayuki HIGUCHI state that:

1. We are joint co-inventors and co-applicants of the subject matter for the invention entitled "ANALYSIS OF AGONIST-ACTIVITY AND ANTAGONIST-ACTIVITY TO CYTOKININ RECEPTOR" as disclosed and claimed in the above-identified patent application, U.S. Application No. 09/918508, filed on August 1, 2001.

2. We are listed as co-authors of a letter to nature present in NATURE, VOL 409, entitled "Identification of CRE1 as a cytokinin receptor from *Arabidopsis*" filed on October 16, 2000 and published on February 22, 2001, and we are joint co-inventors of the subject matter that is disclosed in said letter to nature and disclosed and claimed in the aforementioned patent application.

3. Yukari HASHIMOTO, Motoaki SEKI, Masatomo KOBAYASHI, Tomohiko KATO, Satoshi TABATA and Kazuo SHINOZAKI, are listed as co-authors of said letter to nature present in NATURE, VOL 409, as a general matter of courtesy and are not co-inventors of the subject matter disclosed in said letter to nature or disclosed and claimed in the aforementioned patent application.

We hereby declare that all of the statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like are so made are punishable by fine or imprisonment, or both, under § 1001 of Title 18 of the United States Code and that such willful and false statements may jeopardize the validity of the aforementioned patent application and of any patent issued thereon.

Date: September 14, 2001 Signed: Tatsuo Kakimoto
Tatsuo KAKIMOTO

Date: September 14, 2001 Signed: Tsutomu Inoue
Tsutomu INOUE

Date: September 14, 2001 Signed: Masayuki Higuchi
Masayuki HIGUCHI